# Characterisation of a novel dendritic-like cell in spleen

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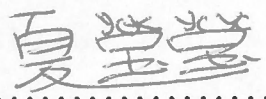
## Statement

Except where otherwise indicated, this thesis is entirely my own work.



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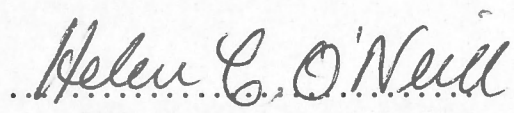

Helen O'Neill

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## Abstract

Dendritic cells (DC) are specialised antigen presenting cells, which induce and control the adaptive immune response. DC mediate a wide range of immune responses including generation of T helper cells, development of cytotoxic T cell responses to viral infection and induction of tolerance. However, DC studies *in vivo* have been hindered by the small numbers of cells available. Various culture systems have been established to overcome this issue. In this lab, a splenic longterm culture (LTC) was established that produces distinct dendritic-like cells (LTC-DC) continuously in the absence of added growth factors or cytokines. Previous studies in this lab identified an *in vivo* equivalent of LTC-DC in murine spleen. This thesis further characterises this novel dendritic-like subset (L-DC). The relationship between L-DC and other known dendritic and myeloid subsets has also been investigated through phenotypic, morphological and functional studies, by comparing gene expression, and by analysis of cell development in mutant mouse models. In order to identify L-DC, it was necessary to redefine splenic dendritic and myeloid subsets.

L-DC have now been characterised with the $CD11b^{hi}CD11c^{lo}MHCII^{-}Ly6C^{-}Ly6G^{-}CD43^{+}CX_3CR1^{+}Siglec\text{-}F^{-}$ phenotype. CD43 expression, lack of MHCII expression, and a low level of CD11c expression best differentiate L-DC from conventional DC (cDC). Like $CD8^{+}$ cDC, L-DC have high capacity for receptor-medicated endocytosis and induce activation and proliferation of $CD8^{+}$ T cells, although not $CD4^{+}$ T cells. L-DC also show capacity to induce an *in vivo* cytotoxic T lymphocyte response equivalent to that induced by $CD8^{+}$ cDC. L-DC are distinguishable from neutrophils by the absence of Ly6G and 7/4 expression, and from eosinophils by lack of Siglec-F expression. L-DC can be distinguished from monocytes by their lack Ly6C expression. Morphological studies revealed L-DC to be mononuclear cells with vacuoles in the cytoplasm, and distinct from other myeloid cell types. All of these findings have identified L-DC as a novel antigen presenting cell subset, distinct from other known dendritic and myeloid subsets in murine spleen.

In this study, resident monocytes were initially analysed according to protocols described in the literature. This led instead to the identification of eosinophils. Resident monocytes are described in literature as $CD11b^{hi}CD11c^{-}Ly6C^{+}Ly6G^{-}CD43^{hi}$ cells. However, since this subset showed low expression of $CX_3CR1$, and expressed high levels of Siglec-F, they were found to be clearly eosinophils. Their morphology was also shown to be consistent with eosinophils, having a multi-lobate nucleus and orange cytoplasmic granules. Resident monocytes were found instead to reside within the $CD11b^{hi}CD11c^{lo}Ly6C^{+}Ly6G^{-}$ subset, verified by high expression of $CX_3CR1$, CD43 and CD115, and an absence of Siglec-F expression. The $CD11c^{lo}$ resident monocyte subset shows morphology similar to inflammatory monocytes, with a bi-lobate nucleus and cytoplasm devoid of granules. Both inflammatory monocytes and neutrophils were gated as $CD11b^{hi}CD11c^{-}Ly6C^{hi}Ly6G^{-}CX_3CR1^{lo}CD43^{lo}$ and $CD11b^{hi}CD11c^{-}Ly6C^{+}Ly6G^{+}CX_3CR1^{-}CD43^{lo}$ cells, as described in the literature. Their identity was also verified morphologically. Splenic macrophages were distinguished in this study as $CD11b^{+}CD11c^{-}Ly6C^{+/-}Ly6G^{-}$ cells which stained for specific macrophage markers including MOMA-1, SIGNR1 and CD68.

Attempts have been made to understand the lineage relationship between L-DC and the other splenic dendritic and myeloid subsets, and to identify new markers for easier delineation of L-DC. RNA was extracted from L-DC, inflammatory monocytes, resident monocytes, eosinophils, $CD8^{+}$ cDC and $CD8^{-}$ cDC. This was prepared for transcriptome analysis employing Mouse Gene 1.0ST genechips (Affymetrix). The L-DC gene expression profile was found to be distinct from that of cDC, and L-DC expressed genes related to myeloid cells like *Itgam*, *Csf1r*, *Emr4* and *Krt80*. However, the L-DC gene expression profile was also found to be distinct from that of resident monocytes and inflammatory monocytes. L-DC specifically expressed genes like *Siglec-e*, *Igsf2*, *CD300ld*, *CD300e* and *CD9*. Overall, gene expression analysis showed that L-DC resembled a myeloid lineage cell having dendritic-like characteristics and function in antigen presentation.

Mouse strains carrying mutations which affect the development of dendritic and myeloid cell types were investigated for changes in L-DC development in

spleen. Antibody staining was used to identify the proportion of dendritic and myeloid cell subsets in mutant versus wild type mice. cDC numbers were found to be significantly reduced in *Flt3L*$^{-/-}$ and *Trif*$^{-/-}$ mice. This indicated that cDC development was dependent on FLT3L and TRIF signalling. In addition, CD8$^{+}$ cDC numbers were significantly reduced in *Batf-3*$^{-/-}$ mice. However, L-DC numbers were not altered in any of the mutant mice, suggesting that L-DC represent a lineage of cells distinct from cDC. Both resident monocyte and inflammatory monocytes developed in lower numbers in *Flt3L*$^{-/-}$ and *GM-CSF*$^{-/-}$ mice, although L-DC were not affected. Resident monocyte numbers were also reduced in *Trif*$^{-/-}$ and *Trif/MyD88*$^{-/-}$ mice, while inflammatory monocytes were found to be reduced in *Batf3*$^{-/-}$ mice. However, L-DC numbers were not affected in any of these mice, confirming that L-DC is a distinct lineage of cells separate from the monocyte lineage and also the cDC lineage.

The representation of DC and myeloid subsets was also investigated in spleens of Booreana mice which carry a non-lethal mutation in *c-Myb* (*c-Myb*$^{E308G}$). *c-Myb* is known to be essential for the development of hematopoietic progenitors in bone marrow. Since L-DC development was not affected in Booreana mice, L-DC may not arise from a bone marrow progenitor. The possibility that L-DC arise from a yolk sac-derived progenitor laid down in spleen during development is now considered. Taken together, these findings indicate that L-DC development occurs independently of *Flt3L*, *Gm-csf*, *Batf3*, *c-Myb* and toll-like receptor (TLR) signaling, so distinguishing the subset from known dendritic and myeloid subsets in spleen.

This study has revealed a novel dendritic-like cell type distinct from known dendritic and myeloid subsets in spleen, and having specialised function in cross-priming CD8$^{+}$ T cells. The findings presented here predict an important role for L-DC in the immune response against blood-borne antigens which enter the splenic microenvironment.

## Table of Contents

## Abbreviations

| | |
|---|---|
| ANU | Australian National University |
| APC | allophycocyanine |
| APC | antigen presentating cells |
| APC-Cy7 | allophycocyanine-Cy7 |
| APF | Australian Phenomics Facility |
| BM | bone marrow |
| Boo | Booreana |
| BSA | bovine serum albumin |
| CCL | chemokine ligand |
| CCR | chemokine receptor |
| CD | cluster of differentiation |
| cDC | conventional dendritic cell |
| CDP | common dendritic progenitor |
| CFSE | 5-(and-6) carboxyfluorescein diacetate succinimidyl esters |
| CLP | common lymphoid progenitor |
| CMoP | common monocyte progenitor |
| CMP | common myeloid progenitor |
| $CX_3CR_1$ | C-X3-C chemokine receptor 1 |
| CXCL | C-X-C chemokine ligand |
| CXCR | C-X-C chemokine receptor |
| Cy7 | indocyanine-7 |
| DC | dendritic cell |
| DMEM | Dulbecco's Modified Eagle's Medium |
| DMSO | dimethyl sulfoxide |
| DTx | diphtheria toxin |
| EDTA | ethylenediaminetetraacetic acid |
| ENU | N-ethyl-N-nitrosourea |
| Eos | eosinophil |
| FACS | fluorescence-activated cell sorting |
| FCS | fetal calf serum |
| FITC | fluorescein isothiocyanate |

| | |
|---|---|
| Flt3(L) | FMS-like tyrosine kinase-3 (ligand) |
| FMOC | fluorescence minus one control |
| FSC | forward scatter |
| GFP | green fluorescent protein |
| GM-CSF | granulocyte/macrophage colony-stimulating factor |
| GMP | granulocyte/macrophage progenitor |
| Granu | granulocyte |
| HEL | hen egg lysozyme |
| HEPES | 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid |
| HSC | hematopoietic stem cell |
| HSPC | hematopoietic stem progenitor cells |
| IFN | interferon |
| IL | interleukin |
| Infl mono | inflammatory monocyte |
| iNOS | inducible nitric oxide synthase |
| JCSMR | John Curtin School of Medical Research |
| KLS | c-kit$^{+}$Lin$^{-}$Sca-1$^{+}$ cells |
| L-DC | *in vivo* equivalent of LTC-DC |
| LPS | lipopolysaccharide |
| LT-HSC | longterm hematopoietic stem cell |
| LTC | longterm culture |
| LTC-DC | longterm culture-derived dendritic cell |
| M-CSF(R) | macrophage colony stimulating factor (Receptor) |
| MACS | magnetic-activated cell sorting |
| MDP | macrophage/dendritic cell progenitor |
| MEP | megakaryocyte-erythroid progenitor |
| MHC | major histocompatibility complex |
| MMM | marginal zone metallophilic macrophage |
| Mo-DC | monocyte-derived dendritic cell |
| MPP | multipotential progenitor |
| MZM | marginal zone macrophage |
| Neu | neutrophil |
| NK | natural killer cell |

| | |
|---|---|
| NO | nitric oxide |
| OVA | ovalbumin |
| p-preDC | plasmacytoid pre-dendritic cells |
| PBS | phosphate buffered saline |
| pDC | plasmacytoid dendritic cell |
| PE | phycoerythrin |
| PE-Cy7 | phycoerythrin-Cy7 |
| PGE2 | prostaglandin E2 |
| PI | propidium iodide |
| pre-cDC | precursor of conventional DC |
| RBC | red blood cell |
| Resi mono | resident monocyte |
| RPM | red pulp macrophage |
| SA | streptavidin |
| sDMEM | supplemented DMEM |
| SE | standard error |
| SSC | side scatter |
| ST-HSC | shortterm hematopoietic stem cell |
| TAP | transporter associated with antigen processing |
| TCR-tg | T cell receptor-transgenic mouse |
| TGF-β | transforming growth factor-β |
| Tip DC | TNF-α/iNOS producing dendritic cell |
| TLR | Toll-like receptor |
| TNF-α | tumor necrosis factor-α |
| Tol DC | tolerogenic DC |
| Treg | regulatory T cell |
| WEHI | Walter and Eliza Hall Institute |
| WMP | white pulp macrophage |
| WT | wildtype |

# Chapter 1

# Introduction

## 1.1 The development of myeloid cell lineages in the mouse model

Myelopoiesis is a regulated process of cell development leading to multiple cell types which contribute to both innate and adaptive immunity. A common myeloid progenitor (CMP) in adult bone marrow gives rise to monocytes/macrophages, dendritic cells (DC), granulocytes and mast cells (Akashi et al., 2000). The granulocyte lineage gives rise to neutrophils, eosinophils, and basophils, each of which provides specific innate immune functions (Ueda et al., 2005). Classification of the monocyte and granulocyte lineages is less well advanced than the DC lineage. While many DC subsets are well defined and can be clearly isolated, the monocyte and granulocyte populations are only partially defined and likely to be heterogeneous.

During embryogenesis, hematopoietic stem cells (HSC) seed fetal liver driving myelopoiesis beginning with the formation of a CMP equivalent (Traver et al., 2001). This gives rise to cells resembling monocytes and granulocytes. Fetal liver and bone marrow precursors have been shown to be similar on the basis of their gene expression profiles (Traver et al., 2001). However, progenitors laid down during embryogenesis can also undergo extramedullary myelopoiesis leading to tissue-specific subsets. There is now an increasing number of reports in the literature of tissue-specific hematopoiesis. For example, microglia are known to self-renew and to proliferate independently of bone marrow-derived precursors (Ajami et al., 2007; Ginhoux et al., 2010). Similarly, Langerhans cells are maintained and replenished by progenitors in skin in the steady-state, and by blood-borne precursors during inflammation (Merad et al., 2002). In addition, a dendritic-like cell type in spleen has been described which derives from endogenous splenic precursors (Tan and O'Neill, 2012; Tan et al., 2011). A recent study has also identified a lineage of yolk sac-derived macrophages present in kidney, pancreas, skin, lungs and spleen tissues (Schulz et al., 2012). These cells appear to develop from progenitors laid down in these tissues during ontogeny.

### 1.1.1 Hierarchy of hematopoietic cell development

Hematopoietic stem cells were first described by the work of Till and McCulloch (Becker et al., 1963; Siminovitch et al., 1963; Till and McCulloch, 1961; Wu et al., 1968). They observed regeneration of the blood system after transplantation of syngeneic bone marrow cells into recipient mice, along with colony formation in recipient mouse spleen. Further analysis of splenic colonies led to the identification of a small subset of donor cells possessing two distinct properties including the ability to self-renew, and the ability to produce erythrocytes and multiple lineages of hematopoietic cells (Becker et al., 1963; Siminovitch et al., 1963; Till and McCulloch, 1961). These two properties are now considered the defining characteristics of a stem cell. Hematopoietic stem cells are the only cells with hematopoietic potential that can differentiate to produce all blood cell types and also divide to give rise to daughter cells without undergoing differentiation.

Hematopoiesis occurs primarily in bone marrow where hematopoietic stem/progenitor cells (HSPC) have been classified as three distinct populations of longterm HSC, shortterm HSC and multipotent progenitors (MPP) (Morrison and Weissman, 1994; Seita and Weissman, 2010; Smith et al., 1991). Initially, HSC were isolated from bone marrow as c-kit$^{+}$Lin$^{-}$Sca-1$^{+}$ (KLS) cells, although this population is now known to be heterogeneous (Seita and Weissman, 2010). Longterm HSC have recently been defined as CD34$^{-}$CD38$^{-}$CD135$^{-}$CD150$^{+}$ KLS cells, while shortterm HSC are CD34$^{+}$CD38$^{+}$CD135$^{-}$CD150$^{+}$ KLS cells. MPP are distinct from longterm and shortterm HSC, since they express CD135, but lack CD150 expression. While both longterm and shortterm HSC have ability to self-renew as well as differentiate into all other lineages, MPP have lost some self-renewal ability (Morrison and Weissman, 1994). The current hierarchial model for hematopoietic cell development now prescribes that MPP differentiate to give lineage progenitors termed common lymphoid progenitors (CLP) (Karsunky et al., 2008; Kondo et al., 1997; Serwold et al., 2009) and common myeloid progenitors (CMP) (Akashi et al., 2000). CLP then give rise to a range of cells including natural killer (NK) cells, Pro-T and Pro-B cells that further differentiate in thymus and spleen respectively, to give T and B cells (Kawamoto et al., 1997).

Further differentiation of CMP gives rise to three distinct progenitors including megakaryocyte/erythrocyte progenitors (MEP), granulocyte/macrophage progenitors (GMP), and the recently described, but disputed macrophage/dendritic cell progenitors (MDP) (Akashi et al., 2000; Diao et al., 2004; Fogg et al., 2006; Liu et al., 2009; Nakorn et al., 2003; Sathe et al., 2014). MEP differentiate further to give platelets and erythrocytes, while GMP differentiate to give granulocytes and macrophages. GMP generate precursors of neutrophils, eosinophils and basophils (Seita and Weissman, 2010), although the nature of the precursor of each granulocyte lineage remains to be investigated.

It has been reported that MDP differentiate to give monocytes and common dendritic progenitors (CDP) (Fogg et al., 2006; Liu et al., 2009; Naik et al., 2013; Varol et al., 2009), generating both cDC and monocytes, with the latter differentiating to give tissue macrophages. Recent evidence now questions the nature of the MDP subset (Sathe et al., 2014), although a downstream CDP is known to exclusively generate conventional DC (cDC) and plasmacytoid DC (pDC) (Liu et al., 2009; Onai et al., 2013; Varol et al., 2007). A monocyte and macrophage committed progenitor downstream of the MDP has now been identified (Hettinger et al., 2013). This common monocyte progenitor (CMoP) gives rise to Ly6C$^{hi}$ monocytes and not DC (Hettinger et al., 2013). However, there is still some uncertainty over the relationship between CDP, CMP and MDP. MDP were originally described as progenitors of cDC since they could replenish splenic DC without the formation of a monocytic intermediate (Varol et al., 2007). However, those experiments utilised bone marrow chimeras generated by reconstitution of lethally irradiated wild type mice with bone marrow from CD11c-diphtheria toxin (DTx) mice (Varol et al., 2007). Irradiation depleted MDP from the host, while DTx treatment depleted macrophages and DC developing from donor bone marrow. However, it is still questionable whether the replenishment of splenic DC occurs from host monocytes under the inflammatory state induced by irradiation of these mice. A recent study now shows that MDP can differentiate to give multiple cell types like DC, macrophages and granulocytes (Sathe et al., 2014). However, DC generated from MDP display the characteristics of monocyte-derived DC (mo-DC) instead of steady-state DC (Sathe et al., 2014). Attempts to identify a bi-potential MDP that give rises to monocytes and steady-state DC were unsuccessful (Sathe at al., 2014). In general,

the development of monocytes and mo-DC is less well characterised than the development of cDC or pDC lineages.

While monocytes are mature when they exit bone marrow and enter blood, cDC precursors (pre-cDC) exit bone marrow, circulating through blood to the spleen and other lymphoid tissues for further differentiation and division (Naik et al., 2006). Early evidence showed $CD11c^{+}MHCII^{-}Ly6C^{+}B220^{+}$ dendritic precursors in bone marrow which give rise to pDC on adoptive transfer (Diao et al., 2004). The $B220^{-}$ variant of this $CD11c^{+}MHCII^{-}Ly6C^{+}$ precursor also gives rise to $CD8\alpha^{-}$ cDC (Diao et al., 2006). Pre-cDC can now be identified in spleen, blood and bone marrow based on a $CD11c^{+}CD43^{+}SIRP\text{-}\alpha^{+}$ phenotype, as well as differentiative potential for cDC (Liu et al., 2009; Naik et al., 2006). Pre-cDC are clearly distinct from monocytes on the basis of their phenotype, morphology and immune function as $CD11c^{inter}CD11b^{lo}F4/80^{lo}SIRP\text{-}\alpha^{inter}$ cells, while monocytes can be differentiated as $CD11c^{-}CD11b^{hi}F4/80^{hi}SIRP\text{-}\alpha^{inter}$ cells (Naik et al., 2006). Pre-cDC also differ from monocytes in that the latter can differentiate in response to M-CSF in culture (Naik et al., 2006). Plasmacytoid DC are now known to develop from $CD11c^{lo}B220^{+}$ circulating immediate precursors (p-preDC) in blood that lodge in spleen under inflammatory conditions to produce pDC (Saunders et al., 1996). Despite these studies, there are still many aspects of dendritic and myeloid cell development which are not well understood.

## 1.2 The dendritic cell lineage

Dendritic cells are the most efficient antigen presenting cells (APC) in the immune system, with unique ability to activate naïve T cells. They are closely aligned with monocytes/macrophages which can also function as APC. Multiple subsets of DC have been identified in both humans and mice (Heath et al., 2004; Naik, 2008), all capable of antigen uptake, processing and presentation for T cell activation. They represent a heterogeneous class of cells with subtypes differing in tissue location, migratory pathway, cell surface marker expression, immunological function and dependence on infection or inflammatory stimulus for their generation (Heath et al., 2004). They are widely distributed throughout the body with distinct subsets present in spleen, mucosa, intestine and epidermis (Heath et al., 2004). Some DC have been classified as migratory, and these cells survey the environment by constant uptake of tissue antigens. In the presence of pathogen-related 'danger signals', these DC mature and migrate to lymph nodes where they present antigens to nearby T cells (Shortman and Naik, 2007). In contrast, lymphoid tissue-resident DC do not migrate, but take up and present incoming antigen to T cells (Shortman and Naik, 2007).

### 1.2.1 Dendritic cell subsets in spleen

Conventional DC (cDC) represent the main DC subset in spleen and have been further classified as functionally distinct $CD8\alpha^+$ and $CD8\alpha^-$ subsets in mice. $CD8\alpha^+$ cDC are distinct as $CD11c^{hi}CD11b^-CD8\alpha^+MHCII^+$ cells, while $CD8\alpha^-$ cDC have a $CD11c^{hi}CD11b^+CD8\alpha^-MHCII^+$ phenotype (Vremec et al., 2000). These subsets differ in immune function, including cytokine production and ability to cross-present antigen (Hochrein et al., 2001). It has been proposed that $CD8\alpha^+$ cDC play a role in the maintenance of tolerance to self-antigens, consistent with their close proximity to T cells in the resting state, and their notable capability for cross-presentation (De Smedt et al., 1996; Steinman et al., 1997). $CD8\alpha^+$ cDC are also the predominant producer of interleukin (IL)-12, a factor important for $CD8^+$ T cell proliferation (Heath et al., 2004). In contrast, $CD8\alpha^-$ cDC have weaker cross-priming ability and are localised mainly in the marginal zone of spleen (De Smedt et al., 1996). Upon stimulation with lipopolysaccharide (LPS), $CD8\alpha^-$ cDC migrate to T

cell areas and secrete inflammatory chemokines (De Smedt et al., 1996).

The pDC is another common DC subset described in spleen (Isaacs and Lindenmann, 1957), existing as a plasmacytoid preDC (p-preDC) in the steady-state. These are non-myeloid cells which are long-lived and circulating, producing high levels of type I interferon after stimulation with viral or other microbial agents (Pelayo et al., 2005). Inflammatory stimuli initiate the conversion of p-preDC into pDC with the production of type I interferons which enhance the function of NK cells, B cells, T cells and DC during antiviral responses. Subsequently, pDC differentiate to give the $CD8\alpha^{+}CD205^{-}$ DC subset, which is distinct from $CD8\alpha^{+}$ cDC and which also regulates T cell function (O'Keeffe et al., 2002). Plasmacytoid DC can be distinguished from other DC subsets and myeloid cells by their $CD11c^{lo}CD11b^{-}B220^{+}$ phenotype, and their high production of type I interferons (Shortman and Naik, 2007). In contrast to other DC that produce type I interferons only upon viral infection, pDC produce type I interferons in response to stimuli that bind Toll-like receptors (Asselin-Paturel et al., 2001; Diebold et al., 2003).

Apart from the clearly defined cDC and pDC subsets, spleen also contains another less well described class of DC termed 'myeloid DC'. This is an umbrella term describing cells with both dendritic and myeloid cell characteristics. During the resting state, myeloid DC comprise mainly tolerogenic DC (TolDC) also known as regulatory DC, which are capable of inducing a tolerogenic T cell response (Steinman et al., 2003). They are phenotypically characterised by expression of SLAM (signalling lymphocyte activation molecule), PD-L1 (programmed cell death ligand-1), DEC-205 and ILT3/ILT4 (immunoglobin-like transcripts 3 and 4) (Maldonado and von Andrian, 2010; Rutella et al., 2006). In addition, TolDC are characterised by constitutive low expression of MHC molecules and co-stimulatory molecules (Morelli and Thomson, 2007). In terms of function, TolDC are capable of promoting the conversion of naïve $CD4^{+}$ T cells into naturally-occurring $CD4^{+}CD25^{+}Foxp3^{+}$ regulatory T cells (Tregs), the induction of Treg1 cells *in vitro* in the presence of cytokines, and the expansion of existing Treg populations (Schmidt et al., 2012; Steinman et al., 2003). Another feature of TolDC is their secretion of indoleamine 2,3-dioxygenase and the suppressive cytokine IL-10 (Morelli and Thomson, 2007; Rutella et al., 2006; Thomson, 2010). TolDC also downregulate the

production of IL-12, a factor essential for effector T cell proliferation and differentiation (Morelli and Thomson, 2007).

Under inflammatory conditions, myeloid DC in spleen can also include mo-DC. Inflammatory stimuli appear to recruit circulating $CD115^{+}Ly6C^{hi}CCR2^{+}$ inflammatory monocytes from blood into spleen where they differentiate to become mo-DC (Geissmann et al., 2008; León et al., 2005, 2007). Different subsets of mo-DC have been observed in different disease models. One example is the TNF/iNOS-producing DC (TipDC) described in *Listeria monocytogenes* infected mice (Serbina et al., 2003). Inflammatory monocytes recruited from blood to *L. monocytogenes* infected spleens, differentiate to become TipDC. These are distinguishable from steady-state cDC on the basis of their unique phenotype as $CD11b^{hi}CD11c^{inter}MAC3^{+}MHCII^{+}$ cells, and their production of high levels of TNF-α and nitric oxide (NO) which induce clearance of microbes from tissue (Serbina et al., 2003). In addition, a mo-DC subset of similar phenotype has been described in the draining lymph nodes of *Leishmania major* infected mice (León et al., 2007). The importance of prostaglandin E in the migration of mo-DC from tissues to T cell zones within lymph nodes has been described (Scandella et al., 2002). Prostaglandin E stimulates expression of CCR7 on mo-DC which binds to CCL19 and CCL21 expressed by lymph node cells (Scandella et al., 2002).

### 1.2.2 Defining characteristic of dendritic cells

Murine splenic DC were first reported by Steinman in 1973. However, it took a decade to establish that DC were novel cells with functions distinct from macrophages. Delineating characteristics of DC are their ability to induce a stronger mixed leukocyte reaction than B cells and macrophages (Steinman and Witmer, 1978), and their ability to cross-prime $CD8^{+}$ T cells. With advances in technology allowing isolation of the rare DC, their phenotypic and functional abilities have been studied in detail. Phenotypically, DC can be distinguished from myeloid cells on the basis of their high expression of CD11c and MHCII.

In line with their high endocytic ability, DC are often located at tissue sites that are constantly exposed to antigen. Exogenous antigens are phagocytosed or

pinocytosed by DC. When phagosomes carrying exogenous antigens fuse with lysosomes containing proteolytic enzymes, antigens are cleaved to give peptides. Newly synthesised MHCII molecules are delivered by vesicular transport to phagolysosomes which contain peptides. Peptides are then loaded on to MHCII molecules for delivery to the cell membrane for subsequent presentation as MHCII/peptide complexes to $CD4^+$ T cells (Groothuis and Neefjes, 2005). Once activated, $CD4^+$ T cells differentiate to become effector T cells that produce cytokines and can activate monocytes for control of infection. In contrast, antigens produced endogenously by infected cells, or defective ribosomal proteins, become tagged with ubiquitin for destruction in the cytoplasm (Sigal et al., 1999). Ubiquitinated proteins are proteolytically cleaved in the cytoplasm to give peptides. The transporter associated with antigen processing selectively allows peptides to enter the endoplasmic reticulum where they are loaded on to MHCI molecules (Groothuis and Neefjes, 2005). The MHCI/peptide complex is then transported to the cell membrane within vesicles for antigen presentation to $CD8^+$ cytotoxic T cells. Dendritic cells can function in the presentation of antigens of both exogenous and endogenous origin.

Some DC subsets can 'cross-present' exogenous antigens acquired from dead or dying cells like tumor cells or virally infected cells on to MHCI molecules for presentation to $CD8^+$ T cells (Huang et al., 1996; Sigal et al., 1999). Cross-presentation is a function largely restricted to $CD8\alpha^+$ cDC (Bedoui et al., 2009; Den Haan et al., 2000; Dudziak et al., 2007; Pooley et al., 2001; Schulz and Reis E Sousa, 2002). The exact mechanism by which exogenous antigens gain access to newly synthesised MHCI molecules, however, remains under investigation. One model involves ER-phagosome fusion whereby the ER dislocon, a protein transporter, participates in cross-presentation at the phagosomal membrane by allowing translocation of peptides from phagosomes into the cytosol (Gagnon, 2002). Another model involves peptides leaking from phagosomes into the cytoplasm, or peptides escaping into cytoplasm through the rupture of the phagosomal membrane, and subsequently being loaded on to MHCI molecules outside the ER (Vyas et al., 2008). While other cell types like neutrophils (Beauvillain et al., 2007) and monocytes/macrophages (Ramirez and Sigal, 2002; Randolph et al., 2008) are thought to function in cross-presentation, these earlier reports remain

unsubstantiated. Indeed definitive information on antigen presenting function is still very dependent on clear and reproducible subset definition and the isolation of pure subsets of cells.

### 1.2.3 Tissue-specific dendritic cells

Apart from spleen, there are multiple secondary lymphoid sites where DC reside. DC play an important role in balancing the induction of tolerance against self antigen during the steady-state with the initiation of an immune response against a pathogen during an inflammatory state. DC can also play different roles in different tissue sites.

#### *1.2.3.1 Epidermal DC*

Skin DC have been extensively characterised. At least five populations of DC can be identified in the skin, distinguishable on the basis of CD103, CD207 and EpCAM expression (Henri et al., 2010; Nagao et al., 2009). The most prominent subset comprises Langerhans cells located in the epidermal layer and identifiable as $CD11b^{inter}CD103^{-}CD207^{hi}EpCAM^{hi}MHCII^{hi}$ cells (Henri et al., 2010; Nagao et al., 2009). The remaining four DC subsets are located within the dermal layer. One subset has been identified as a dermal Langerhans cell on the basis of phenotype. The remaining three dermal DC subsets can be distinguished from Langerhans cells by the absence of EpCAM expression (Nagao et al., 2009). These can be further differentiated into $CD11b^{lo}CD103^{+}CD207^{+}$, $CD11b^{+}CD103^{-}CD207^{-}$ and $CD11b^{-}CD103^{-}CD207^{-}$ subsets (Henri et al., 2010).

Langerhans cells have long been described as the pivotal APC within skin. They capture local antigen and migrate to cutaneous lymph nodes to present antigen directly to T cells. They can also transfer antigen to lymph node resident DC, so inducing an adaptive immune response against the captured antigen. However, recent studies have challenged the role of Langerhans cells, suggesting that $CD11b^{lo}CD103^{+}CD207^{+}$ dermal DC are the true cross-priming subset (Nagao et al., 2009; Seneschal et al., 2014; Stoecklinger et al., 2011). Both Langerhans cells and $CD207^{+}$ dermal DC are capable of cross-presenting OVA to $CD8^{+}$ T cells

(Azukizawa et al., 2003; Waithman et al., 2007). However, others have described the $CD103^+CD207^+$ dermal DC as the main cross-presenting cell type (Bedoui et al., 2009; Henri et al., 2010). One possible explanation for this difference could be contamination of Langerhans cells with dermal DC since cells were not delineated on the basis of CD103 expression (Waithman et al., 2007).

Recent studies have suggested a tolerogenic role for Langerhans cells, whereby Langerhans cells suppress contact hypersensitivity responses through secretion of IL-10 and cognate interaction with $CD4^+$ T helper cells (Igyarto et al., 2009). In a supporting study, Fukunaga et al. (2008) identified $Langerin^-$ dermal DC, and not Langerhans cells, as the main DC subset that induces a contact hypersensitivity response through proliferation of $CD4^+$ T helper cells (Fukunaga et al., 2008). In a parasite study, conditional deletion of Langerhans cells led to enhanced protective immunity against *Leishmania major* infection (Kautz-Neu et al., 2011). This study implicated Langerhans cells as the main subset that suppressed the anti-*Leishmania* immune response through recruitment and expansion of Treg via secretion of IL-10 (Kautz-Neu et al., 2011). In general, Langerhans cells represent tissue-resident DC that participate in immunosuppressive responses, while $CD103^+CD207^+$ dermal DC represent a migratory DC subset that participates in adaptive immune responses.

### *1.2.3.2 Gut mucosal DC*

The gut contains multiple subsets of DC distinguishable by their location, function and phenotype (Johansson and Kelsall, 2005). Within the gut, DC can be first differentiated on the basis of location, namely in Peyer's patches, mesenteric lymph nodes or lamina propria (Johansson and Kelsall, 2005). Peyer's patches and mesenteric lymph nodes are primary sites for the induction of immune responses, while lamina propria are the primary site for development of effector T cells. DC located within these sites have distinct functions, and DC within Peyer's patches and mesenteric lymph nodes include the $CD11b^+CD8\alpha^-$, $CD11b^-CD8\alpha^+$ and $CD11b^-CD8\alpha^-$ subsets (Iwasaki and Kelsall, 2001). Both $CD11b^+CD8\alpha^-$ and $CD11b^-CD8\alpha^-$ DC are migratory cells, while $CD11b^-CD8\alpha^+$ DC are restricted to the T cell zone within lymph nodes (Johansson and Kelsall, 2005). Apart from differences in

location and phenotype, these subsets also have distinct functions. $CD11b^{+}CD8\alpha^{-}$ and $CD11b^{-}CD8\alpha^{-}$ DC are highly endocytic and induce Th2 responses, while $CD11b^{-}CD8\alpha^{+}$ DC are primarily involved in the induction of Th1 responses and the cross-priming of $CD8^{+}$ T cells to confer tolerance against self-antigens (Johansson and Kelsall, 2005).

Lamina propria are the main site for DC contact with food antigen, commensal bacteria and pathogenic bacteria. Hence, DC in lamina propria have distinct function to cope with the environment. They can be distinguished as two main populations of $CD103^{+}$ and $CD103^{-}$ DC (Scott et al., 2011) which induce two distinct T effector cell types. $CD103^{+}CX_3CR1^{-}$ DC are the main subset that migrates from lamina propria to mesenteric lymph node to prime naïve T cells. Conversely, $CD103^{-}CX_3CR1^{+}$ DC lack ability to prime naïve T cells. $CD103^{+}CX_3CR1^{-}$ DC differentiate from CDP and/or pre-cDC and are dependent on granulocyte/ macrophage colony-stimulating factor (GM-CSF) and Fms-like tyrosine kinase 3 ligand (Flt3L) for development (Bogunovic et al., 2009; Scott et al., 2011; Varol et al., 2009). In contrast, $CD103^{-}CX_3CR1^{+}$ DC arise from $Ly6C^{hi}$ inflammatory monocytes which differentiate in the presence of Flt3L and M-CSF (Bogunovic et al., 2009; Scott et al., 2011; Varol et al., 2009). Hence, $CD103^{-}$ DC are thought to be macrophages. Recent studies have shown that $CD103^{+}$ DC possess ability to induce expression of the gut-homing markers CCR9 and $\alpha_4\beta_7$ on naïve T and B cells in mesenteric lymph nodes which aid migration of these cells to lamina propria (Johansson-Lindbom and Agace, 2007; Johansson-Lindbom et al., 2005). In addition, $CD103^{+}$ DC induce the differentiation of $CD4^{+}$ T cells to Tregs via production of IL-10 and TGF-β (Siddiqui and Powrie, 2008; Sun et al., 2007). Tregs are crucial for the maintenance of tolerance towards food and self antigens in the gut. These studies suggest a tolerogenic role for $CD103^{+}$ DC, while $CD103^{-}$ DC prime T cells towards a pro-inflammatory response (Atarashi et al., 2008; Denning et al., 2007).

#### *1.2.3.3 Thymic DC*

T cells develop in thymus where developing thymocytes undergo positive and negative selection before maturing as antigen-specific T cells (Nitta et al., 2008). Most self-reactive thymocytes are removed in thymus via negative selection, a

process crucial for establishment and maintenance of central tolerance (Nitta et al., 2008). Thymic DC are crucial to negative selection, and present self antigens to developing thymocytes causing deletion of T cells which have high self affinity. Three subsets of DC have been described in the thymus: pDC, $CD8^{lo}Sirp\alpha^{hi}$ cDC and $CD8^{hi}Sirp\alpha^{lo}$ cDC (Li et al., 2009; Proietto et al., 2008). $CD8^{hi}Sirp\alpha^{lo}$ cDC develop from early T cell progenitors and are highly efficient at inducing functional Tregs *in vitro* (Proietto et al., 2008). They display a more mature phenotype in terms of expression of MHCII and co-stimulatory molecules compared with other thymic DC subsets (Proietto et al., 2008). In addition, $CD8^{hi}Sirp\alpha^{lo}$ cDC express high levels of CCL17 and CCL22 which attract $CD4^{+}$ thymocytes (Proietto et al., 2008). Recent studies suggest that both pDC and $CD8^{lo}Sirp\alpha^{hi}$ cDC originate extrathymically and continuously migrate into the thymus (Li et al., 2009). $CD8^{lo}Sirp\alpha^{hi}$ cDC can present both self and non-self antigens on MHC molecules. They induce negative selection of thymocytes that strongly bind organ-specific antigens, and positively select T cells for Treg development (Proietto et al., 2008).

Multiple subsets of DC can be identified in different tissue sites, and each DC subset has a specialised role in tissues. This reflects compartmentalisation and a mechanism to regulate the immune response efficiently.

## 1.3 Myeloid subsets in spleen

Dendritic cells are located mainly within the white pulp region of spleen where immune responses against blood-borne antigens and pathogens are initiated, while myeloid cells are primarily located within the red pulp region. White pulp comprises three regions: the periarteriolar lymphoid sheath (PALS) which is the T-cell zone, B cell follicles, and the marginal zone (Cesta, 2006). The PALS can be further divided to give the inner PALS which comprises mainly $CD4^+$ T cells, some $CD8^+$ T cells, interdigitating DC and migrating B cells, while the outer PALS contains macrophages (Mebius and Kraal, 2005). The marginal zone is strategically situated at the interface of the red pulp and PALS for screening blood-borne antigens and pathogens. It contains a large reservoir of resident myeloid cells as APC that participate in mounting an immune response against blood-borne antigens.

### 1.3.1 Splenic macrophages

Four subsets of macrophages have been described for spleen. Two of these are located within the marginal zone, namely marginal zone metallophilic macrophages and marginal zone macrophages (Mebius and Kraal, 2005). Marginal zone metallophilic macrophages can be visualised by staining with the MOMA-1 monoclonal antibody and are located near the PALS and B cell follicles (Mebius and Kraal, 2005). Recent studies have described marginal zone metallophilic macrophages as essential for induction of cytotoxic T cell responses against blood-borne antigens (Backer et al., 2010). Marginal zone metallophilic macrophages concentrate antigens in the marginal zone via SIGLEC1 which interacts with sialic acid residues on pathogens (Mebius and Kraal, 2005). Subsequently, marginal zone metallophilic macrophages phagocytose pathogens and transfer antigens to $CD8^+$ cDC which then mount a cytotoxic T cell response against the pathogen. They can also induce tolerance against self antigens (Backer et al., 2010; Ravishankar et al., 2014). In addition, there is increased production of CCL22 in marginal zone metallophilic macrophages following administration of apoptotic cells (Ravishankar et al., 2014). CCL22 can recruit and activate Tregs to suppress inflammatory responses elicited by apoptotic cells (Ravishankar et al., 2014).

Marginal zone macrophages are located closer to the red pulp and are characterised by SIGNR1 expression (Mebius and Kraal, 2005). These macrophages express a number of pattern recognition receptors like Toll-like receptor (TLR) type 1 scavenger receptor MARCO, and the C-type lectin SIGNR1. They play an important role in the clearance of microorganisms (Kang et al., 2003). SIGNR1 efficiently binds polysaccharide antigen on several microorganisms like *Mycobacterium tuberculosis* (Kang et al., 2003), and can bind antigens expressed on viruses (Oehen et al., 2002). In addition, marginal zone macrophages have been reported to play a role in the retention and activation of marginal zone B cells (Karlsson et al., 2003).

The third subset is the tangible body macrophages within the white pulp of spleen. These can be distinguished from other macrophages by their phenotype as $CD11b^{-}F4/80^{-}CD68^{+}$ cells (Noel et al., 2007) and their involvement in the phagocytosis of apoptotic B cells generated during germinal center reactions. A final subset is the red pulp macrophages. These are distinct by F4/80 expression ($CD11b^{+}F4/80^{+}CD68^{+}$) which distinguishes them from marginal zone metallophilic macrophages, marginal zone macrophages and tangible body macrophages, all of which are $F4/80^{-}$ (Noel et al., 2007). Red pulp macrophages are involved mainly in the clearance of old or damaged red blood cells and the recycling of heme groups.

### 1.3.2 Splenic monocytes

Monocytes are thought to develop in bone marrow from a common myeloid/dendritic cell progenitor (MDP) which exists only in bone marrow (Liu et al., 2009; Yona et al., 2013). MDP progeny continuously migrate into blood via a CCR2-dependent mechanism (Geissmann et al., 2008). When monocytes enter tissues they terminally differentiate to give macrophages. Monocyte-derived DC may also develop in lymphoid sites in response to pathogen invasion and inflammatory stimuli (Randolph et al., 1998).

In general, two subsets of monocytes have been identified in blood: the $CX_3CR1^{lo}Ly6C^{hi}$ inflammatory monocytes, and the $CX_3CR1^{hi}Ly6C^{-}$ resident monocytes (Geissmann et al., 2008; Yona et al., 2013). Inflammatory monocytes

home to sites of infection where they differentiate to give TipDC (Geissmann et al., 2003), while resident monocytes home to non-inflammatory sites and are thought to be precursors of macrophages resident in normal tissues, e.g. liver and spleen (Geissmann et al., 2003). However, information on the function of resident monocytes, and whether or not they differentiate to give macrophages within tissues, is still debatable. Contradictory evidence suggests that tissue-specific macrophages, e.g. Kupffer cells in liver, lung alveolar macrophages and splenic macrophages, are maintained through adulthood without replenishment from blood monocytes (Davies et al., 2013; Hashimoto et al., 2013; Schulz et al., 2012). This raises questions about the function of resident monocytes and whether all tissue-specific macrophages are monocyte-derived. Indeed evidence for a yolk sac-derived lineage of macrophages argues for development of tissue-specific macrophages from progenitors endogenous to tissues (Schulz et al., 2012).

A reservoir of undifferentiated monocytes resident in spleen was recently identified (Swirski et al., 2009). Phenotypically, morphologically and transcriptomically these were shown to resemble blood monocytes. Under inflammatory conditions, both inflammatory monocytes and resident monocytes are selectively mobilised from spleen to the site of inflammation. Splenic inflammatory monocytes were found to clear damaged tissues, while splenic resident monocytes promoted wound healing (Swirski et al., 2009). In addition, spleen was found to deploy large numbers of monocytes to injury sites faster than from bone marrow. Deployment of a reservoir of splenic monocytes was hypothesised as a mechanism for faster initiation of an immune response. The potential role of spleen in myelopoiesis was therefore predicted from this study.

### 1.3.3 Yolk sac-derived macrophages

Tissue macrophages can develop from either bone marrow-derived or yolk sac-derived progenitors. During primitive hematopoiesis, yolk sac-derived progenitors give rise to hematopoietic cells including erythrocytes and macrophages (Palis and Yoder, 2001). Yolk sac-derived macrophages then migrate to and colonise other fetal organs. Several studies have highlighted how tissue-specific macrophages appear to have a yolk sac origin (Ginhoux et al., 2010; Hashimoto et al., 2013;

Schulz et al., 2012). In particular, lineage tracing studies using *Runx1*$^{Cre/wt}$:*Rosa26*$^{R26R\text{-}eYFP}$ mice have confirmed that adult microglial cells derive from yolk sac precursors (Ginhoux et al., 2010). A similar tracing study using *Runx1*$^{Cre/wt}$:*Rosa26*$^{R26R\text{-}eYFP}$ mice identified yolk sac-derived macrophages as CD11b$^{lo}$F4/80$^{hi}$ cells, while bone marrow-derived macrophages were CD11b$^{hi}$F4/80$^{lo}$ cells (Schulz et al., 2012). Unlike bone marrow-derived macrophages, yolk sac-derived macrophages develop independently of the *c-Myb* transcription factor and the Flt3L growth factor (Schulz et al., 2012). Their development is instead dependent on the transcription factor PU.1 (Schulz et al., 2012). A recent study suggests a third possible origin for tissue macrophages from fetal monocytes. Parabiosis studies and BrdU labelling experiments showed that circulating monocytes contribute minimally to alveolar macrophage development (Guilliams et al., 2013). Instead, adoptive transfer experiments showed that alveolar macrophages derive from fetal monocytes (Guilliams et al., 2013). Similarly, Langerhans cells have been shown to derive from both yolk sac-derived macrophages and fetal monocytes (Hoeffel et al., 2012).

### 1.3.4 Splenic granulocytes

Granulocytes in spleen are mainly localised in the red pulp region with some evident in transition through the marginal zone as blood-borne cells flowing into red pulp (Nolte et al., 2000). In general, granulocytes comprise neutrophils, eosinophils, basophils and mast cells, although neutrophils are the major subset within spleen (Nolte et al., 2000). Neutrophils are key components of the inflammatory response, recruiting APC, transporting antigen, and controlling T-cell expansion and differentiation (Beauvillain et al., 2007). They can be distinguished from other leukocytes on the basis of their phenotype as CD11b$^{hi}$CD11c$^{-}$Ly6G$^{+}$F4/80$^{-}$ MHCII$^{-}$ cells (Abadie et al., 2005; Beauvillain et al., 2007). Recently, it was shown that neutrophils from inflammatory peritoneal exudates could cross-prime CD8$^{+}$ T-cells both *in vivo* and *in vitro* (Beauvillain et al., 2007; Tvinnereim et al., 2004). While these studies demonstrated cross-priming capability, this response occurred only under inflammatory stimulus, and so may not be reflective of neutrophils in steady-state spleen.

Other granulocyte subsets can also be detected in blood and tissues, although in lower frequency. Eosinophils are indicative of parasitic infections, allergies and some rare diseases (Stone et al., 2010). They are distinguishable from other granulocytes on the basis of their unique morphology with a bi-lobate nucleus and highly condensed chromatin, as well as the presence of orange-stained granules in the cytoplasm (Muniz et al., 2012; Walsh, 2013). In terms of cell surface markers, eosinophils express a range of markers including CD32, CD89, CR3, CD88, CCR1, CCR3, Siglec-F, IL-3R, IL-5R, GM-CSFR and multiple TLR (Stone et al., 2010). Upon activation, eosinophils release proinflammatory granules containing cationic proteins and cytokines. At least three types of cationic proteins are present in eosinophilic granules which can disrupt cell membranes. They also possess RNAse activity to target parasites and virus infections (Stone et al., 2010; Walsh, 2013), and can catalyse the oxidation of NO and halides into products which are toxic for parasites and host cells (Stone et al., 2010; Walsh, 2013).

As with eosinophils, mast cells are detected mainly in tissues. Mast cells develop from $CD34^{+}$ progenitors in bone marrow and then circulate into tissues sites where they mature to form functional mast cells (Metcalfe, 2008; Stone et al., 2010). They are distinguishable from other granulocytes on the basis of ckit, FcεR1 and FcγRIIa expression (Stone et al., 2010). In particular tissue sites, mast cells may upregulate other markers like FcγRI, prostaglandin receptor EP2, complement C3a and C5a receptors, as well as various interleukin, chemokine and toll-like receptors (Metcalfe, 2008; Stone et al., 2010). Upon activation, mast cells release granules containing mediators like histamine, proteases and proteoglycans as well as cytokines. Histamine plays an important role in inflammatory and allergic responses, and recruits leukocytes to sites of inflammation, as well as regulating mucous secretion and smooth muscle contraction (Jutel et al., 2006; Metcalfe, 2008). Proteases break down ligands like fibrinogen, fibronectin, and the complement C3 molecule (Stone et al., 2010). They can also activate fibroblasts, encourage accumulation of inflammatory cells, and potentiate histamine-induced airway bronchoconstriction (Stone et al., 2010). Lastly, mast cells secrete a range of cytokines like tumour necrosis factor (TNF)-α, IL-3, IL-5, IL-6, IL-10, IL-13 and GM-CSF which can regulate the immune response (Metcalfe, 2008). IL-3, IL-5 and GM-CSF are crucial for eosinophil development.

Basophils share many traits with mast cells including expression of FcεR1, secretion of T helper 2 cytokines, and the release of histamine upon activation (Karasuyama et al., 2011; Stone et al., 2010). However, basophils can be distinguished from mast cells by their rapid and large secretion of IL-4 and IL-13 (Karasuyama et al., 2011). In addition, basophils mature in bone marrow and remain within the circulatory system, rather than in tissue sites (Karasuyama et al., 2011). Due to their low numbers and their similarity with mast cells, basophils have been neglected and considered redundant in immune responses. Recent studies have highlighted the unique role of basophils in the regulation of immunity. Basophils act as initiators in the development of IgE-mediated chronic allergic inflammation in the skin, unlike mast cells that act as effectors in the inflammatory response (Karasuyama et al., 2009; Mukai et al., 2005). Basophils also help drive the Th2 response against allergens via IL-4 secretion (Hida et al., 2005; Oh et al., 2007).

## 1.4 Dendritic cell development in spleen

### 1.4.1 Dendritic cell culture systems

Since DC are a rare cell type, *in vitro* culture systems have been developed to generate large numbers of cells for studies of function. There have been two main culture systems developed for the generation of DC, each giving cells with distinct function (Randolph et al., 2008). The first utilises a cytokine cocktail of GM-CSF, TNF-α and IL-4, which stimulates myeloid precursors and monocytes to give mo-DC (Sallusto and Lanzavecchia, 1994; Xu et al., 2007). The second method utilises Flt3L which stimulates proliferation and differentiation of bone marrow derived pre-DC to give cDC and pDC (Brawand et al., 2002; Naik et al., 2005; Xu et al., 2007). The GM-CSF/IL-4 culture system is an attractive model for generating large numbers of DC. However, it produces only mo-DC expressing CD11c and MHC-II, which have properties reflective of DC generated under inflammatory conditions. These cells do not reflect steady-state or immature DC. Evidence has shown that GM-CSF levels are low in resting mice but increase dramatically during inflammation, consistent with evidence that cDC and pDC are present at normal levels in spleens of GM-CSF$^{-/-}$ mice (Vremec et al., 1997).

Flt3L is a known proliferative factor for DC (Brasel et al., 2000), and a growth factor for hematopoietic progenitors. It also mobilises hematopoietic progenitors and stem cells *in vivo* (Merad and Manz, 2009). *Flt3L*$^{-/-}$ mutant mice have lower numbers of DC in spleen, thymus and lymph nodes, emphasising the overall importance of Flt3L in DC development (McKenna et al., 2000). It was recently shown that *in vitro* grown Flt3L-induced cDC resemble the *in vivo* subsets of CD8α$^{+}$ cDC and CD8α$^{-}$ cDC in spleen (Naik et al., 2005). Cell phenotypes of CD24$^{hi}$CD11b$^{lo}$CD172α$^{lo}$ and CD24$^{lo}$CD11b$^{hi}$CD172α$^{hi}$ reflect the CD8α$^{+}$ cDC and CD8α$^{-}$ cDC subsets described in murine spleen respectively (Brawand et al., 2002; Naik et al., 2006).

The Flt3L culture system is distinct from the GM-CSF/IL-4 culture system in that it generates steady-state and not inflammatory DC. GM-CSF-induced DC and Flt3L-induced DC appear to derive from distinct precursors (Xu et al., 2007). When

the CD11b$^{hi}$Ly6C$^{+}$Ly6G$^{-}$ inflammatory monocyte population from bone marrow was cultured with GM-CSF, cells produced resembled a TipDC as a CD11b$^{hi}$CD11c$^{lo}$Mac-3$^{+}$MHC-II$^{+}$ cell subset (Geissmann et al., 2003). No cDC production was induced upon culture of inflammatory monocytes with Flt3L. Inflammatory monocytes in bone marrow appears to be GM-CSF-responsive DC precursors, and these appear to be the *in vitro* counterpart of TipDC (Auffray et al., 2009).

### 1.4.2 Longterm splenic cultures produce dendritic-like cells

Another method for DC production *in vitro* involves splenic longterm cultures (LTC) which produce a distinct dendritic-like cell type (O'Neill et al., 2004). These 'LTC-DC' arise from spleen-derived progenitors maintained over long periods in contact with murine splenic stromal cells (Wilson and O'Neill, 2004; Wilson et al., 2000). Splenic stroma maintained within LTC supports continuous but restricted development of only this cell type, without the addition of cytokines like GM-CSF, TNF-α, IL-4 or Flt3L to cultures. The continuous production of cells within LTC is consistent with the maintenance of self-renewing progenitors or stem cells within the stromal layer (Wilson and O'Neill, 2004). Recent studies have confirmed that progenitors are maintained in LTC by endothelial-like stromal cells unique to spleen (Despars and O'Neill, 2006). Hematopoietic cells form foci of proliferating cells above the stroma, and continuously shed dendritic-like cells into the medium.

### 1.4.3 Further characterisation of LTC-DC

Non-adherent cells collected from LTC comprise a minor population of small precursors/progenitors, and a major population of large cells reflecting immature splenic DC (Quah et al., 2004). The non-adherent small cells represent a heterogeneous population expressing markers of hematopoietic progenitors like cKit, Sca-1 and Thy-1, with subsets of large-sized cells expressing CD11b, CD11c, CD117 and CD80 having weak endocytic ability (Quah et al., 2004). LTC-DC production was achieved when a sorted small cell population was cultured over the splenic stromal cell line STX3, derived from a LTC that had lost hematopoietic cells with passage over time (Wilson and O'Neill, 2004). This study confirmed the

presence of self-renewing progenitors within the small cell population produced in LTC (Wilson and O'Neill, 2004). In contrast, the large, non-adherent cells produced reflected LTC-DC, a subset of immature myeloid dendritic-like cells. They had the cell surface phenotype of $CD11b^{hi}CD11c^{lo}CD8\alpha^{-}MHCII^{-}CD80^{+}CD86^{+}$ cells (Quah et al., 2004). The absence of MHCII and CD40 expression distinguished LTC-DC from DC derived by culture of precursors with inflammatory cytokines. Hence, LTC-DC differ from mo-DC generated in the GM-CSF/IL-4 culture system (Sallusto and Lanzavecchia, 1994; Xu et al., 2007), and from cDC or pDC generated in the Flt3L culture system (Brawand et al., 2002; Naik et al., 2005; Xu et al., 2007).

A notable characteristic of LTC-DC is their ability to cross-prime $CD8^{+}$ T cells (Tan and O'Neill, 2010). LTC-DC demonstrated superior ability to cross-prime $CD8^{+}$ T cells when compared with freshly isolated $CD11c^{+}$ splenic DC. However, LTC-DC lack ability to activate $CD4^{+}$ T cells, consistent with their $MHCII^{-}$ phenotype. LTC-DC are therefore distinct from other splenic DC subsets or DC derived in other *in vitro* culture systems which activate $CD4^{+}$ T cells.

### 1.4.4 Identification of an *in vivo* equivalent of L-DC

An *in vivo* equivalent of LTC-DC, namely 'L-DC', has been identified in murine spleen based on an equivalent phenotype, cross-priming capability, and inability to activate $CD4^{+}$ T cells (Tan and O'Neill, 2010). Like LTC-DC, L-DC are large cells, which express the same $CD11b^{hi}CD11c^{lo}CD8^{-}MHCII^{-}$ phenotype. The *in vitro* cross-priming capability of L-DC was found to be comparable with that of $CD8^{+}$ cDC (Heath et al., 2004; Tan and O'Neill, 2010). While the cross-presentation capacity of $CD8^{+}$ cDC increases further after LPS activation, L-DC were found to be strong activators of $CD8^{+}$ T cells assessed *in vitro*, only with LPS activation (Tan and O'Neill, 2010). Evidence for an *in vivo* cell type equivalent to LTC-DC, gives physiological relevance to the *in vitro* derived cell type named LTC-DC. The possibility that spleen produces an endogenous APC with function specific to the splenic environment, and to blood-borne pathogens and antigens, is under further consideration.

Preliminary studies have shown that L-DC are well positioned compared with other splenic myeloid and DC subsets for uptake of blood-borne antigens (Tan and O'Neill, 2010). *In vivo* antigen priming prior to isolation of APC subsets, followed by *in vitro* assays of T cell activation, showed that L-DC were superior to $CD8^+$ cDC and monocytes in uptake and presentation of antigen (Tan and O'Neill, 2010). L-DC have also been shown to be capable of cross-priming $CD8^+$ T cells in adoptive transfer experiments (Tan and O'Neill, 2010), and may therefore represent an important cross-presenting subset in spleen, especially under inflammatory conditions. To date, they have been identified as $FSC^{hi}$ cells within the $CD11b^{hi}CD11c^{lo}CD8^-MHCII^-$ splenic subset. However, this subset is heterogeneous and further characterisation is needed to delineate L-DC more specifically.

### 1.4.5 Other splenic stroma-based culture systems

Several other groups have also reported production of dendritic-like cells in spleen stroma culture systems. One group described the differentiation of bone marrow-derived DC to give $CD11b^{hi}CD11c^{lo}CD8^-CD80^{hi}CD40^{hi}MHCII^{lo}$ DC (diff DC) in cultures over spleen-derived stroma (Tang et al., 2006; Zhang et al., 2004). In addition, this group also identified an *in vivo* counterpart of diff DC on the basis of cell surface phenotype. Both *in vitro* and *in vivo* diff DC were shown to secrete nitric oxide and to inhibit $CD4^+$ T cells, so functionally resembling regulatory DC (Tang et al., 2006; Zhang et al., 2004). In addition, *in vitro* generated diff DC have been shown to induce apoptosis in $CD4^+$ T cells via Fas ligand induced IFN-γ and NO production (Xu et al., 2012). *In vitro* generated diff DC also reprogrammed $CD4^+$ T memory cells to give immunosuppressive T memory cells which suppress $CD4^+$ T cell activation and proliferation via IL-10 secretion (Xu et al., 2011). Lastly, *in vitro* generated diff DC also induced differentiation of splenic B cells to give regulatory B cells via IFN-β secretion and CD40 ligand signalling (Qian et al., 2012). The relationship between regulatory DC and L-DC is not known, and is yet to be identified.

Another group generated IL-10 producing DC with a $CD11b^{hi}CD11c^{lo}CD45RB^+$ phenotype by culturing $CD117^+Lin^-$ bone marrow progenitors over splenic stroma comprising mainly fibroblastic cells (Svensson and

Kaye, 2006; Svensson et al., 2006). These $CD11c^{lo}CD45RB^{+}$ DC display regulatory functions including suppression of mixed leukocyte reactions, differentiation of $CD4^{+}$ Th cells into IL-10 producing Treg cells, as well as antigen-specific tolerance following adoptive transfer (Svensson and Kaye, 2006). These $CD11c^{lo}CD45RB^{+}$ DC resembled the regulatory $CD11c^{lo}CD45RB^{hi}$ DC described by Wakkach et al. (2003) in terms of their phenotype and secretion of IL-10. However, in Wakkach et al. (2003), a GM-CSF and TNF-α culture system was used to generate DC from bone marrow progenitors, which then further differentiated to give regulatory DC ($CD11c^{lo}CD45RB^{hi}$) upon addition of IL-10 (Wakkach et al., 2003). Currently, the development and role of regulatory DC is not well understood, and more than one pathway for development appears to be possible.

### 1.4.6 Spleen as a site for hematopoiesis

While hematopoiesis occurs mainly in the bone marrow in adults, spleen can act as a secondary site for hematopoiesis (Wolber et al., 2002). Extramedullary hematopoiesis has also been described in the yolk sac, liver and spleen during fetal development before bone marrow formation (Lux et al., 2008; Palis and Yoder, 2001; Tavassoli, 1991; Wolber et al., 2002). In response to pathogens, HSC in spleen can differentiate to give increased numbers of APC and phagocytes (Morita et al., 2011; Shatry and Levy, 2004). The capacity of spleen stroma to support hematopoiesis, and particularly myelopoiesis, in the absence of additional growth factors or cytokines was first described by Ni and O'Neill (1997). Subsequent studies have identified and isolated stromal cell lines that support extramedullary hematopoiesis *in vitro* with production of dendritic-like cells (O'Neill et al., 2014; Periasamy and O'Neill, 2013; Periasamy et al., 2013). Early *in vitro* studies are now supported by the identification of an *in vivo* equivalent of LTC-DC (Tan and O'Neill, 2010). All of these studies support a model whereby spleen stroma supports HSPC differentiation to give a specific dendritic-like cell type. This appears to be unique to spleen and to display distinct functional capacity in activation of $CD8^{+}$ but not $CD4^{+}$ T cells.

## 1.5 Hypothesis and Aims

Progress in identification and characterisation of an *in vivo* equivalent of LTC-DC has led to the identification of a putative subset of L-DC (Tan et al., 2011). However this subset is heterogeneous and requires further delineation. Given the availability of new markers which delineate splenic dendritic and myeloid cell subsets and the availability of mouse strains mutated for specific genes, it is now possible; 1) to further delineate and characterise L-DC in relation to other cell lineages both phenotypically and genotypically, 2) to investigate the functional relationship between L-DC and other splenic dendritic and myeloid subsets, and 3) to assess the lineage origin of L-DC in terms of requirement for cytokines and growth factors during development.

The following hypothesis is therefore investigated: **L-DC reflect a lineage of dendritic-like cells distinct from known subsets of dendritic and myeloid cells present in spleen in terms of phenotype, gene expression and function in T cell activation.**

Specific aims:

1. To distinguish a L-DC candidate subset from splenic subsets of resident monocytes, inflammatory monocytes, eosinophils, neutrophils, cDC and pDC, on the basis of cell surface marker expression.

2. To investigate the function of a putative L-DC subset in terms of ability to activate $CD8^+$ and $CD4^+$ T cells, and to cross-present antigen in comparison with other well defined APC subsets in spleen.

3. To investigate the development of L-DC in spleens of mouse strains mutated for genes which regulate the development and function of other known subsets of dendritic and myeloid cells.

4. To profile gene expression in a candidate L-DC subset purified from spleen, and to identify genes specifically expressed in L-DC which could encode new markers for

cell identification. The relationship of L-DC with known DC and monocyte subsets in spleen will be assessed on the basis of gene expression profiles.

# Chapter 2

# Materials and Methods

## 2.1 Murine cell development

### 2.1.1 Animals

Mice were obtained from several different sources. Specific pathogen-free C57BL/6J, C57BL/6.Tg(TcraTcrb)1100Mjb (OT-I), C57BL/6.SJL/J.OT-II.CD45.1 (OT-II), C57BL/6-Tg(CAG-OVA)916Jen:WehiAnu (Act-mOVA), C57BL/6-*MyD88*$^{-/-}$ (MyD88$^{-/-}$) and C57BL/6-*TRIF*$^{-/-}$ (*TRIF*$^{-/-}$) mice were obtained from the John Curtin School of Medical Research (JCSMR, Australian National University (ANU), and used at 4-6 weeks of age. C57BL/6-*Flt3L*$^{tm1Imx}$ (*Flt3L*$^{-/-}$) mice (Taconic Farms Inc., NY, USA) were purchased from the Biomedical Research Facility, University of Western Australia (Perth, Australia), while B6.129P(Cg)-*Ptprc*$^{a}$*Cx3Cr1*$^{tm1Litt}$/LittJ (*Cx3Cr1-GFP*) and C57BL/6-*MyD88*$^{-/-}$*TRIF*$^{-/-}$ (*MyD88*$^{-/-}$ *TRIF*$^{-/-}$) mice were purchased from the Walter and Eliza Hall Institute (WEHI: Parkville, Victoria, Australia). C57BL/6-*Csf2*$^{tm1Ard}$ (*GM-CSF*$^{-/-}$) mice were obtained from the Ludwig Institute for Cancer Research (Melbourne, Australia). Lastly, C57BL/6-*c-Myb*$^{E308G}$ (*Booreana*) and C57BL/6-129S-*Batf*$^{tm1.1Kmm}$ (*Batf-3*$^{-/-}$) mice were provided through the courtesy of Peter Papathanasiou (JCSMR, ANU) and Ian Cockburn (JCSMR, ANU), respectively. Mice were housed and handled according to protocols approved by the Animal Experimentation Ethics Committee at the ANU, and sacrificed by cervical dislocation.

### 2.1.2 Isolation and culture of cells

Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 22.2mM D-glucose, 13μM folic acid, 27μM L-asparagine, 5.5mM L-arganine HCL, 10% heat inactivated Fetal Calf Serum (FCS) (JRH Biosciences: Lenexa, Kansas, USA), 10mM Hepes (JRH Biosciences), 2mM L-glutamine (JRH Biosciences), 17.1μM streptomycin (JCSMR), 100U penicillin and 50μM 2-mercaptoethanol (BDH Ltd.: Poole, England) per litre of medium. This is referred to as supplemented DMEM (sDMEM). Cells were maintained in 5% $CO_2$ in air with 97% humidity at 37°C.

To prepare a suspension of lymphoid cells, dissected spleen or thymus was pressed through a fine mesh sieve. Cells were resuspended into 5mL red blood cell (RBC) lysis buffer (140mM NH4Cl, 17mM Tris base) for 5 minutes at 20°C. Cells were then washed twice in sDMEM by centrifugation (5 minutes, 300g), and counted after the final wash. For preparation of bone marrow (BM), the femur and tibia of a C57BL/6J mouse were dissected and the marrow flushed out by injection of sDMEM using a syringe and 26G needle. Cells were dissociated, cleared of RBC, washed and resuspended as for splenocytes. For T cell activation studies, cells were isolated from spleen, and enriched for T cells by depletion of myeloid and B cells.

For counting, cells were stained with 0.4% Trypan blue/saline (Gibco BRL: Grand Island, NY, USA). Unstained cells were counted using a haemocytometer to determine the number of live cells/mL for adjustment of cell concentration.

### 2.1.3 Fractionation of cells

#### 2.1.3.1 Depletion of T and B cells from spleen

Dendritic and myeloid cells were enriched from dissociated whole spleen via negative depletion of T and B cells using magnetic bead separation and MACS® technology (Miltenyi Biotec: Auburn, California, USA). T and B cell depletion was performed by exposing cells to specific antibody: 0.2µg biotinylated anti-Thy1.2 antibody/$10^8$ cells (T cells) and 0.25µg biotinylated anti-CD19 antibody/$10^8$ cells (B cells) in 1 mL. Cells were pelleted (4°C, 5 minutes,300g) and the supernatant aspirated. They were then resuspended at $10^8$ cells/mL of MACS labelling buffer (2mM EDTA/0.5% Bovine Serum Albumin (BSA) in Phosphate-Buffered Saline (PBS)) and incubated on ice for 25 minutes. After incubation, cells were washed twice with 5mL of MACS labelling buffer, with centrifugation (4°C, 5 minutes, 300g) between each wash. Cells were resuspended in MACS labelling buffer ($10^8$ cells/mL), followed by addition of 20µl of anti-biotin microbeads/$10^8$ cells (Miltenyi) and incubation for 25 minutes on ice. The washing step was repeated once, and cells were resuspended in 500µl of MACS labelling buffer. T and B cells were depleted by running cells through LS columns (Miltenyi) in a SuperMACS II

Separation Unit (Miltenyi), washing thrice with 3mL of MACS buffer, and collecting unbound cells as flow-through.

### 2.1.3.2 Isolation of $CD11c^+$ cells from spleen

CD11c is a surface marker expressed by many DC subsets. To isolate $CD11c^+$ DC, splenocytes ($10^8$ cells/mL) were incubated for 25 minutes on ice with 20μL of anti-CD11c magnetic microbeads (Miltenyi)/$10^8$ cells and washed once with 5mL MACS labelling buffer. Cells were resuspended in 500μL MACS labelling buffer. Columns were placed in the SuperMacs II magnet and pre-conditioned by washing with MACS labelling buffer using 500μL for MS columns and 3mL for LS columns, with discard of effluent. For positive selection of $CD11c^+$ cells, the cell suspension was loaded on to the column and unlabelled cells collected as the effluent. To collect $CD11c^+$ labelled cells, the column was washed thrice with MACS labelling buffer and the effluent discarded. Labelled cells were eluted with 1mL MACS labelling buffer for MS columns, and 5mL for LS columns, either allowing elution by gravity, or by using a column plunger to apply pressure. Cells were then sedimented at 300g and counted.

### 2.1.3.3 Purification of T cells

For enrichment of $CD4^+$ and/or $CD8^+$ T cells from OT-I or OT-II mice, spleen or mesenteric lymph nodes (MLN) were aseptically removed and processed as described in Section 2.2. Cells were pelleted ($4^0$C, 5 minutes, 300g), and then resuspended in hybridoma culture supernatant (CSN) containing antibody to deplete macrophages, B cells and $MHCII^+$ antigen presenting cells (APC). The CSN antibody cocktail was prepared by addition of 50μL anti-CD11b (clone: M1/70), anti-B220 (RA3-6B3) and anti-$IA^{b/k}$ (TIB120) per $10^7$ cells. For depletion of $CD4^+$ or $CD8^+$ T cells, 50μL of anti-CD4 (GK1.5) or anti-CD8 (53-6.7) CSN per $10^7$ cells was included in the antibody cocktail. Cells were incubated with antibodies for 25 minutes on ice and washed twice with MACS labelling buffer. Cells were then incubated with sheep anti-rat Ig Dynabeads® (Invitrogen Dynal: AS, Oslo, Norway) (50uL beads/$10^7$cells) at $4^0$C for 25 minutes with rotation prior to placing cells in a

magnetic particle separator (Dynal®) for 2 minutes. Supernatant containing unbound T cell subsets was transferred into a new tube.

An alternative method was used for isolation of $CD4^+$ and/or $CD8^+$ T cells from OT-I or OT-II mice. Splenocytes were prepared as described in Section 2.2, then enriched for T cells via depletion of B cells as described in Section 2.3.1. Enriched splenocytes were then stained with specific antibodies against Thy1.2, Vα2, and CD4 (for OT-I isolation) or CD8 (OT-II isolation), as described in Section 2.4.2. The population of interest was isolated by sorting following flow cytometeric analysis of fluorochrome-labelled antibody binding to cells as described in Section 2.4.6.

### 2.1.4 Flow Cytometry

#### 2.1.4.1 Antibodies

Antibodies were purchased as affinity purified, flurochrome or biotin conjugates. These are listed in Table 2.1. Isotype control antibodies and secondary fluorescent conjugates are shown in Table 2.2. All antibodies were titrated before use on splenocytes or BM cells to determine the concentration giving minimum saturation binding to cells.

#### 2.1.4.2 Cell staining procedure

For single colour analysis, $5x10^5 - 1x10^6$ cells were sedimented into the wells of a 96-well polystyrene microtitre plate (Corning: New York, USA) using centrifugation (4°C, 10 minutes, 300g). Supernatant was discarded by flicking the plate, and cells resuspended in anti-CD16/32 (FcBlock : Biolegend, USA) at 5μg/mL with incubation on ice for 15 minutes. The plate was centrifuged (4°C, 5 minutes, 300g), and supernatant aspirated. Cells were resuspended in $25\mu L/10^6$ cells of diluted primary antibody, and incubated on ice for 25 minutes. Cells were washed twice using $150\mu L/10^6$ cells FACS (Fluorescence-activated cell sorting) buffer (1% FSC, 0.1% sodium azide in DMEM) with centrifugation (5 minutes, 300g) between each wash. If necessary, cells were then resuspended in $25\mu L/10^6$ cells of diluted

**Table 2.1. Primary antibodies used in flow cytometry**

| Specificity | Conjugation [a] | Clone of origin | Isotype | Final Concentration (mg/mL) | Source[b] |
|---|---|---|---|---|---|
| 7/4 | Biotin | 7/4 | Rat $IgG_{2a}$ | 0.1 | 2 |
| CD115 | PE | AFS98 | Rag $IgG_{2a,\kappa}$ | 0.5 | 5 |
| CD11b | Biotin | M1/70 | Rat $IgG_{2b,\kappa}$ | 0.5 | 5 |
| CD11b | FITC | M1/70 | Rat $IgG_{2b,\kappa}$ | 1.25 | 5 |
| CD11b | Pe-Cy7 | M1/70 | Rat $IgG_{2b,\kappa}$ | 0.2 | 5 |
| CD11c | APC | N418 | Hamster IgG | 0.2 | 5 |
| CD16/32 (FcBlock) | - | 93 | Rat $IgG_{2a}$ | 0.5 | 5 |
| CD172a (SIRPα) | FITC | P84 | Rat $IgG_1$ | 0.5 | 3 |
| CD19 | Biotin | eBio1D3 | Rat $IgG_{2a,\kappa}$ | 0.5 | 4 |
| CD24 | PE | M1/69 | Rat $IgG_{2b,\kappa}$ | 0.5 | 4 |
| CD4 | FITC | GK1.5 | Rat IgG2b | 0.5 | 5 |
| CD40 | FITC | HM40-3 | Hamster IgM,κ | 0.5 | 5 |
| CD43 | Alexa488 | IBII | Rat $IgG_{2a}$ | 1.25 | 4 |
| CD43 | APC-Cy7 | IBII | Rat $IgG_{2a}$ | 0.5 | 4 |
| CD68 | Biotin | FA-11 | Rat $IgG_{2a}$ | 0.1 | 2 |
| CD69 | PE-Cy7 | H1.2F3 | Armenian Hamster IgG | 0.1 | 5 |
| CD8α | FITC | 53-6.7 | Rat $IgG_{2a,\kappa}$ | 1.25 | 3 |
| CD8α | PE | 53-6.7 | Rat $IgG_{2a,\kappa}$ | 0.2 | 5 |
| CD8α | Alexa 700 | 53-6.7 | Rat $IgG_{2a,\kappa}$ | 0.5 | 4 |
| cKit (CD117) | PE | 2B8 | Rat $IgG_{2a,\kappa}$ | 0.2 | 5 |
| F4/80 | Biotin | CI:A3-1 | Rat $IgG_{2b}$ | 1.25 | 4 |
| Foxp3 | PE | MF-14 | Mouse $IgG_{1,\kappa}$ | 0.2 | 4 |
| $IA^b$ MHCII | Biotin | 25-9-17 | Mouse $IgG_{2a,\kappa}$ | 0.5 | 3 |
| $IA^b$ MHCII | FITC | AF6-120.1 | Mouse $IgG_{2a,\kappa}$ | 0.5 | 3 |
| Ly6C | FITC | AL-21 | Rat IgMκ | 0.25 | 3 |
| Ly6C | Pacific Blue | HK1.4 | Rat IgMκ | 0.5 | 4 |
| Ly6G | Biotin | 1A8 | Rat $IgG_{2a,\kappa}$ | 1.25 | 4 |

| | | | | | |
|---|---|---|---|---|---|
| Ly6G | PE | 1A8 | Rat $IgG_{2a,\kappa}$ | 0.2 | 3 |
| Mac 3 | PE | M3/84 | Rat $IgG_{1,\kappa}$ | 0.5 | 5 |
| Mannose receptor | FITC | MR5D3 | Rat igG2a | 0.1 | 2 |
| MOMA-1 | Biotin | MOMA-1 | Rat $IgG_{2a}$ | 0.4 | 1 |
| Siglec F | PE | E50-2440 | Rat $IgG_{2a,\kappa}$ | 0.2 | 3 |
| SIGN-R1 | Biotin | ER-TR9 | Rat IgM | 0.1 | 2 |
| TCR-Vα2 | APC | B20.1 | Rat $IgG_{2a}$ | 0.2 | 5 |
| Ter119 | Biotin | TER-119 | Rat $IgG_{2b,\kappa}$ | 0.5 | 4 |
| Thy1.2 | Biotin | 30-H12 | Rat $IgG_{2b,\kappa}$ | 0.5 | 5 |
| $IA^b$ MHCII | Pacific Blue | M5/114.15.2 | Rat $IgG_{2b,\kappa}$ | 0.5 | 4 |

a APC, allophycocyanin; Alexa488, Alexa Fluor ® 488; Alexa700, Alexa Fluor ® 700; FITC, fluorescein isothiocyanate; PE, phycoerythrin; PE-Cy7, phycoerythrin-Cy7

b 1: Abcam (Cambridge, Massachusetts, USA), 2:AbD Serotec (Oxford, UK), 3: BD Pharmingen, 4: Biolegend (San Diego,CA,USA), 5:eBioscience (Parkville, VIC, Australia)

**Table 2.2. Isotype antibodies and secondary reagents used in flow cytometry**

| Specificity | Conjugation [a] | Clone of origin | Isotype | Source [b] |
|---|---|---|---|---|
| Hamster IgG | APC | eBio299Arm | Hamster IgG | 5 |
| Mouse $IgG_{2a}$ | Biotin | eBM2a | Mouse $IgG_{2a}$ | 5 |
| Rat $IgG_1$ | PE | RTK2071 | Rat $IgG_1$ | 4 |
| Rat $IgG_{2a}$ | PE, FITC | R35-95 | Rat $IgG_{2a}$ | 3 |
| Rat $IgG_{2b}$ | PE-Cy7, FITC | eB149/10H5 | Rat $IgG_{2b}$ | 5 |
| Rat $IgG_{2b}$ | PE | RTK4530 | Rat $IgG_{2b}$ | 4 |
| Rat IgM | Biotin | RTK2118 | Rat IgM | 4 |

Secondary Reagent

| Specificity | Conjugation[a] | Final Concentration (ug/mL) | Source[b] |
|---|---|---|---|
| Streptavidin | APC-Cy7 | 0.2 | 5 |
| Streptavidin | FITC | 25 | 5 |
| Streptavidin | PE | 0.5 | 3 |

a APC, allophycocyanin; APC-Cy7, allophycocyanin-Cy7; FITC, fluorescein isothiocyanate; PE, phycoerythrin; PE-Cy7, phycoerythrin-Cy7

b 1: Abcam (Cambridge, Massachusetts, USA), 2:AbD Serotec (Oxford, UK), 3: BD Pharmingen, 4: Biolegend (San Diego,CA,USA), 5:eBioscience (Parkville, VIC, Australia)

secondary antibody and incubated for 25 minutes on ice. The washing procedure was repeated after staining, and cells resuspended in 50μL FACS buffer before transfer to a FACS tube (Corning) for flow cytometric analysis using a LSRII or Fortessa flow cytometer (Becton Dickinson: Franklin Lakes, New Jersey, USA). Prior to analysis, propidium iodide (PI: 1μg/mL) was added for dead cell discrimination. PI was not used in single colour staining controls, or when permeablised cells were stained with antibody.

For multicolour analysis, cells were aliquoted and incubated with FcBlock as described above. Cells were then resuspended in a mixture of n primary antibodies where antibodies were used at *n* times the concentration used in single colour staining. The same staining procedure was used as described for single colour staining. Fluorochromes were selected to avoid interference in terms of emission wavelengths.

### 2.1.4.3 Intracellular staining

Cells were washed with 1mL cold PBS and then resupended into 0.25% paraformaldehyde/cold PBS, with incubation on ice for an hour. Cells were then sedimented (4°C, 5 minutes, 300g), resuspended into 1mL 0.2% Tween20, incubated at 37 °C for 15 minutes, and then pelleted by centrifugation (4°C, 5 minutes, 300g). Cells were washed with 1mL of cold PBS, and then incubated with 25ul of 1μg/mL PE-conjugated anti-Mac3 antibody on ice for 30 minutes. After incubation, cells were washed twice with 1mL 0.1% Tween20 with centrifugation (300g, 5 minutes) between each wash. Cells were then resuspended in 50μl of FACS buffer and transferred to FACS tubes for flow cytometric analysis.

### 2.1.4.4 CFSE labelling of cells

Cells were stained with 5-(and 6-) carboxyfluorescein diacetate succinimidyl ester (CFSE: Molecular Probes: Eugene, Oregon, USA) for quantitation of proliferation in terms of the dilution of stain with each cell division measured flow cytometrically as a decrease in fluorescence. To stain cells, enriched T cell suspensions were washed and resuspended in 1mL/$10^7$cells CFSE labelling buffer

(PBS/0.1%BSA). CFSE was added at a final concentration of 2.5μM and vortexed immediately upon addition to ensure uniform cell labelling. Cells were incubated at $37^0$C for 10 minutes followed by addition of 5 volumes of cold complete medium with incubation on ice for 5 minutes to quench labelling. Cells were pelleted ($4^0$C, 5 minutes, 300g), and washed twice with complete medium.

### 2.1.4.5 Marker expression analysis using flow cytometry

Flow cytometry was used to identify cell surface marker expression on cells by measuring the binding of fluorescent-labelled antibodies. In each experiment, cell labelling involved multiple (multicolour) antibodies specific for markers of interest. Compensation was performed on single colour samples (stained with one antibody) to avoid the emission of one fluorochrome interfering with the detection of another. In addition, voltage parameter and event counts were also set before sample acquisition. Background binding was monitored using isotype control antibodies corresponding to each fluorochrome-conjugated antibody used. Fluorescence Minus One Controls (FMOC) were used to set gates for analysis of positively stained cells. Live cells were detected by exclusion of propidium iodide (PI, 1μg/mL), which intercalates in the DNA of dead or dying cells. Analysis was performed using FACSDiva Software (Becton Dickson) and Flow Jo software (Tristar: Phoenix, Arizona, USA). Between $5x10^4$ and $1x10^6$ cells were analysed for each sample, and data was collected for each flurochrome used, along with forward scatter (FSC) and side scatter (SSC) data.

### 2.1.4.6 Cell sorting

Cell populations were isolated by sorting following flow cytometric analysis of fluorochrome-labelled antibody binding. Cells were prepared as described in Section 2.4.1 and all incubation and washing steps were performed in sodium azide-free FACS buffer. After a final wash prior to sorting, cells were filtered through a 70μm nylon cell strainer (Becton Dickinson) into a 5mL polystyrene tube (Becton Dickinson) for removal of cell clumps. Sorted populations were collected in complete medium for cell culture and functional assays.

## 2.2 Murine cell function

### 2.2.1 Endocytosis

The capacity of cells to take up antigen was assessed by *in vivo* measurement of endocytosis. Ovalbumin conjugated to FITC (OVA-FITC) was delivered intravenously to mice at 1mg/mouse according to a time course. Mice were sacrificed and their spleens harvested at the end of the timed study. Splenocytes were RBC lysed and enriched via T and B cell depletion as described in Section 2.2 and 2.3.1. After depletion, cells were collected for antibody staining and flow cytometric analysis.

### 2.2.2 Activation of $CD8^+$ T cells

The cross-presentation capacity of isolated DC and APC subsets was measured by the ability of antigen-pulsed cells to induce proliferation in purified $CD8^+$ T cells isolated from OT-I T cell receptor-transgenic (TCR-Tg) mice specific for ovalbumin ($OVA_{257-264}$/H-$2K^b$). Freshly isolated splenic $CD11c^+$ DC were prepared as control APC as described in Section 2.3.2. DC and APC were plated in 96 well culture plates in $10^7$/mL of sDMEM, pulsed with 10μg/mL of OVA or HEL (Hen Egg Lysozyme) overnight (8-12 hrs), then washed by centrifugation (300g, 5 minutes) twice. Some DC or APC were activated via addition of lipopolysaccharide (LPS, 10μg/mL) for 8-12 hrs.

An alternative source of DC or APC were subsets isolated from transgenic Actm-OVA mice, which constitutively express OVA on the cell membrane via the actin promoter. In Actm-OVA mice, APC express OVA antigen in the context of MHCI via the cross-presentation pathway after uptake and clearance of dead cells.

$CD8^+$ T cells were purified from spleens of OT-I mice as described in Section 2.3.3, and labeled with CFSE to measure proliferation flow cytometrically as described in Section 2.4.4. CFSE-labelled $CD8^+$ T cells were added to DC and APC at various ratios ($10^4$-$10^5$cells/well) in a total volume of 200μL. Cells were collected after 4 days, stained with anti-CD8 antibody, and proliferation determined flow

cytometrically in terms of CFSE dilution and blastogenesis of cells, i.e increase in size.

### 2.2.3 Activation of $CD4^+$ T cells

The ability of isolated DC and APC subsets to process and present antigen to $CD4^+$ T cells was assessed in terms of the proliferation of $CD4^+$ T cells isolated from OT-II TCR-Tg mice specific for ovalbumin ($OVA_{323-339}$/I-$A^b$). DC and APC subsets were isolated from Actm-OVA mice.

$CD4^+$ T cells were sorted from spleens of OT-II mice as described in Section 2.4.6, and CFSE labelled for flow cytometric measurement of proliferation as described in Section 2.4.4. CFSE-labelled $CD4^+$ T cells were added to OVA-expressing DC and APC at various ratios ($10^4$- $10^5$cells/well) in a total volume of 200μL. Cells were collected after 4 days and proliferation determined flow cytometrically in terms of CFSE dilution and blastogenesis of cells, i.e increase in size.

### 2.2.4 Effector function of $CD8^+$ T cells

The ability of APC to induce a cytotoxic T lymphocyte (CTL) response was investigated via lysis of target cells. Targeted cell lysis was measured using a fluorescent target array (FTA) developed by Ben Quah (JCSMR, ANU).

On Day 0, $CD8^+$ and $CD4^+$ T cells were isolated from OT-I and OT-II TCR-Tg mice respectively, as described in Section 2.3.3. Equal numbers of $CD8^+$ and $CD4^+$ T cells were delivered into individual host mice (C57BL/6J) via intravenous injection (3.5 x $10^6$ cells/mouse). At one hour after delivery of T cells, various APC subsets sorted from Actm-OVA mice were also delivered into host mice. Three concentrations of APC were used: 90,000, 9000 or 900 cells. The effector function of activated $CD8^+$ T cells was measured on Day 7 via lysis of peptide-pulsed target cells adoptively transferred intravenously on Day 6. Target cells were isolated from B6.SJL spleen as described in Section 2.3.1 and labelled with CFSE, Cell Trace Violet (CTV) (both from Molecular Probes, Invitrogen) and Cell Proliferation Dye

(CPD) (eBioscience). Splenocytes were resuspended at 0.5-2 x $10^8$ cells/mL in 20°C sDMEM and labelled with a final concentration of 0, 400, 2250 and 12,500nM of each dye. Labelled splenocytes were pulsed with SIINFEKL (SIIN), SIINKL (N6), SIIGFEKL (G4) and EIINFEKL (E1) respectively, for an hour at 37°C. All peptides were synthesised at the Biomolecular Research Facility (JCSMR, ANU). Pulsed target cells (2.5 x $10^7$) were delivered intravenously into host mice one day prior to harvesting host splenocytes for FACS analysis of target cells. Specific killing of target cells was determined by the following formula.

$$\% \text{ specific killing} = \left[ 1 - \left( \frac{\text{Targets}^{+\text{peptide}}_{\text{primed}} / \text{Targets}^{+\text{nil}}_{\text{primed}}}{\text{Targets}^{+\text{peptide}}_{\text{naive}} / \text{Targets}^{+\text{nil}}_{\text{naive}}} \right) \right] \times 100$$

### 2.2.5 Immunohistochemistry

#### 2.2.5.1 Giemsa staining

Cell staining with Giemsa was employed for morphological delineation of cells within sorted cell populations. Cells ($10^3$ – $10^6$ in 200μL) were pelleted on to a glass slide using a cytospin centrifuge (900rpm, 5 minutes). Cells were fixed in methanol for five seconds, then stained in a two-step procedure with CliniPure staining solution 1 (0.25% Eosin YO/Soresen buffer, pH 6.8) followed by CliniPure staining solution 2 (0.25% methylene blue polychrone /Soresen buffer, pH 6.8) (HD Scientfic; NSW, Australia) for five seconds at each step. Excess staining solution was rinsed off under running water, and slides dried before mounting. A non-aqueous mounting agent (Depex, Fluka Analytical: Buchs, Switzerland) was used to prevent leaching of dye from stained cells. Photographs were taken with a LEICA DFC digital camera connected to a LEICA brightfield inverted microscope (LEICA Microsystems, Wetzlar, Germany).

## 2.3 Gene expression analysis

Mouse Gene 1.0ST genechips (Affymetrix, Santa Clara, CA, USA) were used for transcriptome analysis of gene expression in splenic myeloid and DC subsets. The aim of the experiment was to determine differentially expressed genes between the subsets, to better delineate L-DC, and to better characterise this subset in terms of lineage, function and cell surface phenotype.

### 2.3.1 Preparation of RNA

Splenic myeloid and DC subsets were isolated and sorted from C57BL/6J mice as described in Sections 2.3.1 and 2.4.6. Sorted subsets were washed thrice with PBS to remove any trace of sorting medium.

RNA was extracted from sorted cell subsets using an RNeasy mini kit (Qiagen: Clifton Hill. VIC, Australia). The cell pellet was resuspended in 350μL of RLT buffer containing 3.5μL of β-mercaptoethanol and vortexed for 5 minutes to lyse cells. 350μL of 70% ethanol was added to the cell lysate which was transferred into the RNeasy column, avoiding any cell debris. The RNeasy column with collection tube was centrifuged in a Microfuge 18 centrifuge (Beckman Coulter) for 15 seconds at 9200g, and the flowthrough discarded. 700μL of RW1 buffer was added to the column and the column centrifuged for 15 seconds at 9200g. The flowthrough and collection column were discarded after the spin. The RNeasy column was fitted with a new collection tube. 500μL of RPE buffer was added to the RNeasy column, and centrifuged for 15 seconds at 9200g. The flowthrough was discarded and the column washed with another 500μL of RPE followed by a 2 minute spin at 9200g. The flowthrough and collection tube were discarded at the final spin. The RNeasy column was placed in a new collection tube and 30μL of RNAse free water added. The column was allowed to stand for 5 minutes before centrifugation at 9200g for 1 minute. RNA was collected as flowthrough and stored at -20°C.

### 2.3.2 RNA quality and yield

A preliminary assessment of RNA quality and yield was determined spectrophotometrically using an ND-100 spectrophotometer (Nanodrop: Wilmington, DE, USA). MilliQ water was used to calibrate the spectrophotometer at absorbance of 260nm and 280nm. The yield of RNA was determined by reading A260nm, such that a reading of 1.0 at A260nm indicates a concentration of 40µg/mL of RNA. The quality of RNA was determined by the ratio of $A_{260}/A_{280}$, with value of 1.8 to 2.1 reflecting high purity. A lower ratio indicates protein contamination, while a higher ratio indicates degradation of RNA.

A more sensitive method of determining RNA quality and yield employed the RNA 6000 Pico Chip (Aglient Technologies: Waldbronn, Germany) followed by analysis on an Agilent 2100 Bioanalyser. The chip was loaded with a gel and dye mixture followed by a conditioning solution. An indicator was added to all wells, prior to addition of samples and the molecular weight ladder. Readout from the bioanalyser gives RNA purity, quality and concentration.

### 2.3.3 Microarray analysis of gene expression

RNA was prepared from sorted myeloid and DC subsets, labelled and hybridised on to Mouse Gene 1.0ST genechips (Affymetrix) by Kaiman Peng (Biomolecular Resource Facility, JCSMR: ANU, Canberra, Australia) according to the Applause WT-Amp ST and WT-Amp Plus ST RNA Amplification Systems protocol published online by the NuGEN technologies (San Carlos, CA, USA): (http://www.nugeninc.com/nugen/index.cfm/products/apl/applause-rna-amplification-systems/). Initially, first stand cDNA was generated from the RNA sample using a unique first strand DNA/RNA chimeric primer mix and reverse transcriptase (RT). The DNA portion of the primer hybridises either to the 5' end of the poly A sequence or randomly across the transcript. RT extends the 3' end of each primer generating first strand cDNA. The resulting cDNA/RNA hybrid molecule contains a unique RNA sequence at the 5' end of the cDNA strand. Subsequently RNA within the cDNA strand was fragmented to allow DNA polymerase to synthesise a second strand of DNA that complements the 5' unique sequences from

the first strand primer. Amplification of cDNA was performed with the SPIA amplification kit developed by NuGEN technologies. The cDNA samples were fragmented and labelled according to the FL-Ovation™ cDNA Biotin Module V2 protocol (NuGEN technologies), followed by hybridisation on to the genechip. The genechip was washed and stained using the fluidics station (Affymetrix), prior to scanning and analysis using a GeneArray® Scanner (Affymetrix).

### 2.3.4 Analysis of microarray data

Scanned images of genechips were processed using Partek (St. Louis, Missouri, USA). Data files were prepared containing probeset numbers, gene descriptions, signal values and p-values in text file format. Data files were subsequently exported into Excel (Microsoft: Redmond, WA, USA) for further processing. The annotation of probesets was performed using the Gene Ontology caterogies of 'Biological Process', 'Cellular Component' and 'Molecular Function', available on the Affymetrix website (www.Affymetrix.com). Further Analysis of data involved selection of datasets using Excel on the basis of defined analysis criteria, for example, identification of genes specific to subsets, and identification of genes common to subsets. In addition, dendrograms, bivariate plots, and heatmaps were prepared using R project (http://www.r-project.org/).

Hierarchical clustering analysis employed a set of dissimilarities for clustering gene expression amongst dendritic and myeloid subsets. Initially, each subset was assigned to its own cluster of gene expression and then the algorithm proceeded iteratively, at each stage joining the two most similar subsets, and continuing until all subsets were clustered on the basis of their gene expression. At each stage, distances between subsets were recomputed by the Lance–Williams dissimilarity update formula (Sibson, 1973). Hierarchical clustering analysis is presented as dendrograms, sometime on heatmaps.

Pairwise comparison of total gene expression by subsets was presented as bagplots which are a generalisation of the 1 dimensional box plot (Rousseeuw et al., 1999). The inner polygon is called the bag and contains approximately 50% of the observations. Outliers are defined as points lying outside the polygon formed by

inflating the bag by a factor of 3, which is called the fence (not drawn in the bagplot). The outer depicted polygon is called the loop and is the convex hull of all points which are not outliers.

## 2.4 Statistical analysis

Data have been presented as mean ± standard error for sample size n. For sample size $n \leq 5$, where normal distribution cannot be assumed, the Wilcoxon Rank Sum test was used to test significant ($p \leq 0.01$ or 0.05). Where a normal distribution could be assumed, Students' *t*-test was used to determine significance ($p \leq 0.01$ or 0.05).

# Chapter 3

# Identification of *in vivo* equivalent of longterm culture dendritic-like cells

## 3.1 Introduction

Dendritic cells (DC) are a rare cell type, making up only ~1% percent of the total spleen cell population. Studies on DC have been very much limited by their low cell number. To counter this problem, many groups have established *in vitro* culture systems that generate DC from bone marrow progenitors under the influence of growth factors like Flt3 ligand (Flt3L) and granulocyte/macrophage colony stimulating factor (GM-CSF) (Kingston et al., 2009). In contrast, O'Neill et al. (2004, 2011) have established a stroma-dependent culture system from spleen that produces dendritic-like cells continuously without the addition of growth factors or cytokines. This culture system is termed a longterm culture (LTC), and the dendritic-like cells produced have been called 'LTC-DC' (O'Neill et al., 2004).

Production of LTC-DC is dependent on the presence of splenic stromal cells. The stromal layer within LTC produces all growth factors and cytokines needed to induce LTC-DC production from precursors/progenitors continuously for at least a year (O'Neill et al., 2004; Wilson and O'Neill, 2003). Isolation of stroma from LTC has led to the establishment of the STX3 splenic stromal cell line, comprising a mixture of both fibroblastic-like cells and stromal cells with more endothelial-like morphology (Despars and O'Neill, 2006; O'Neill et al., 2004). The LTC system represents a model stromal niche which supports restricted hematopoiesis. It is hypothesised that splenic stroma in LTC maintains hematopoietic stem/progenitor cells (HSPC) and produces factors that induce differentiation and production of LTC-DC.

Phenotypic and functional characterisation of cells produced in LTC has led to the identification of LTC-DC as $FSC^{hi}CD11b^{hi}CD11c^{lo}CD8^{-}MHCII^{-}$ cells with strong ability to endocytose antigen and to cross-prime $CD8^{+}$ T cells (O'Neill et al., 2004; Quah et al., 2004). However, LTC-DC are distinct from other 'described' DC in that they lack ability to activate $CD4^{+}$ T cells. On the basis of cell phenotype and function, LTC-DC resemble immature, myeloid DC. Many studies have been conducted to establish their relationship with known subsets of DC present in murine spleen but they remain a novel, unrelated cell type (O'Neill et al., 2004; Periasamy et al., 2009; Petvises and O'Neill, 2014; Tan and O'Neill, 2010, 2012). An *in vivo*

equivalent population has been partially defined, and distinguished both phenotypically and functionally from known DC subsets (Tan and O'Neill, 2012).

This chapter describes LTC-DC produced *in vitro* and demonstrates their functional potential. Further investigation of an *in vivo* counterpart cell has been conducted, based on the properties of LTC-DC. This study involves initial analysis of 'L-DC' as a novel subset in spleen in relation to other known splenic dendritic and myeloid subsets.

## 3.2 Results

### 3.2.1 Phenotypic identification of LTC-DC

LTC were established from whole dissociated spleens of C57BL/6J mice. Cultures were maintained for at least 6 weeks before analysis of cell production. At this time, established LTC were characterised by semi-adherent small progenitor cells, adherent fibroblastic-like cells and other stroma, and large non-adherent dendritic-like cells (Figure 3.1 A). Non-adherent cells were collected from the supernatant of LTC and stained with antibodies for flow cytometric analysis of cell surface markers. Large LTC-DC were first delineated on the basis of size as a $FSC^{hi}$ and $SSC^{mid}$ population (Figure 3.1 B). This population was further delineated on the basis of CD11b and CD11c expression as a uniform population of $CD11b^{hi}CD11c^{lo}$ cells. Further staining of this gated population revealed cells with a $MHCII^{-}CD8^{-}$ $B220^{-}$ phenotype. In addition, cells produced in LTC were negative for Ly6C and Ly6G expression. In summary, LTC-DC reflect a population of $FSC^{hi}SSC^{mid}CD11b^{hi}CD11c^{lo}CD8^{-}MHCII^{-}Ly6C^{-}Ly6G^{-}B220^{-}$ cells with dendritic-like morphology and phenotype.

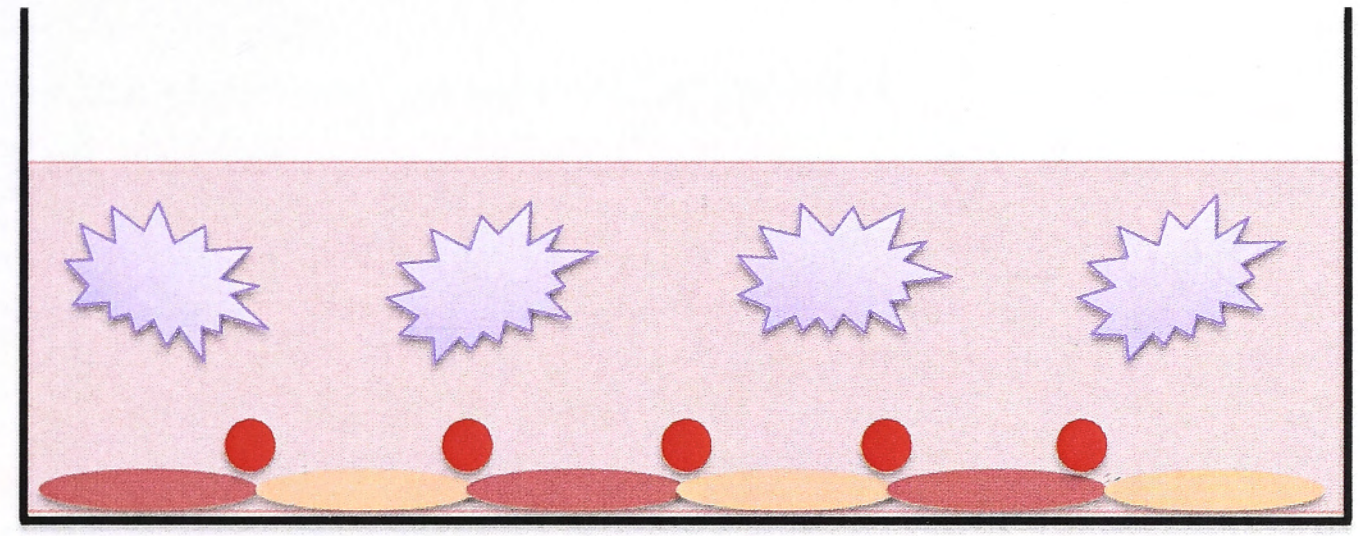
### 3.2.2 Identification of an *in vivo* equivalent subset of LTC-DC

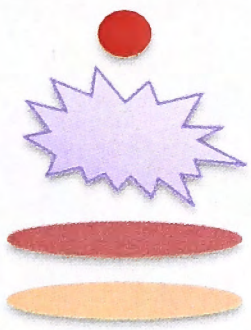
The phenotype of LTC-DC was used in identification of an *in vivo* 'L-DC' subset. Splenocytes were depleted of T and B cells using magnetic bead technology and then stained with a cocktail of labelled antibodies as described above. For flow cytometry, cells were stained with propidium iodide (PI) for initial gating of $PI^{-}$ live cells. Cells were then gated on the basis of CD11b and CD11c expression to delineate L-DC candidates, conventional DC (cDC) and myeloid cells. Conventional DC were initially gated as $CD11b^{+/-}CD11c^{hi}$ cells, and then further delineated on the basis of CD8 and MHCII expression (Figure 3.2). In line with the literature, $CD8^{+}$ cDC are $CD11b^{-}CD11c^{hi}CD8^{+}MHCII^{+}$ cells (26.7%), while $CD8^{-}$ cDC are $CD11b^{+}CD11c^{hi}CD8^{-}MHCII^{+}$ cells (61.1%) (Shortman and Liu, 2002). Myeloid cells were gated as $CD11b^{hi}CD11c^{-}$ cells. In general, the myeloid population comprises granulocytes, monocytes and activated macrophages (Iwasaki and Akashi, 2007). All of these cell types except macrophages have been described as $MHCII^{-}$

**Figure 3.1 Phenotypic characterisation of longterm culture (LTC)-derived cells.**

A splenic LTC system has been established that continuously produces a dominant cell type resembling DC. A) The LTC comprises adherent fibroblastic-like and other stromal cells, small progenitor cells and LTC-DC. The adherent stromal cells provide a niche that supports the development of LTC-DC from hematopoietic progenitors in spleen. B) Phenotypic characterisation of LTC-DC. Non-adherent cells were collected from culture and stained with 2 cocktails of antibodies. The first included antibodies specific for CD11b (M1/70, PE-Cy7), CD11c (N418, APC), MHC-II (25-9-17, Biotin), CD8 (53-6.7, PE) and B220 (RA3-6B2, FITC). The second included antibodies specific for CD11b (M1/70, PE-Cy7), CD11c (N418, APC), MHC-II (25-9-17, Biotin), Ly6C (Al-21, FITC) and Ly6G (1A8, PE). Prior to analysis, cells were stained with propidium iodide (PI, 1μg/ml) for gating live ($PI^-$) cells. Large cells were gated by forward scatter (FSC) versus side scatter (SSC) analysis. LTC-DC were gated as $CD11b^{hi}CD11c^{lo}MHCII^-$ cells for further analysis. Independent control stainings of splenocytes were used to confirm antibody specificity and activity (data not shown). Gates were set based on isotype controls and numbers in gates represent % specific binding.

A





Progenitors
LTC-DC
Stromal cells
Fibroblastic-like cells

B

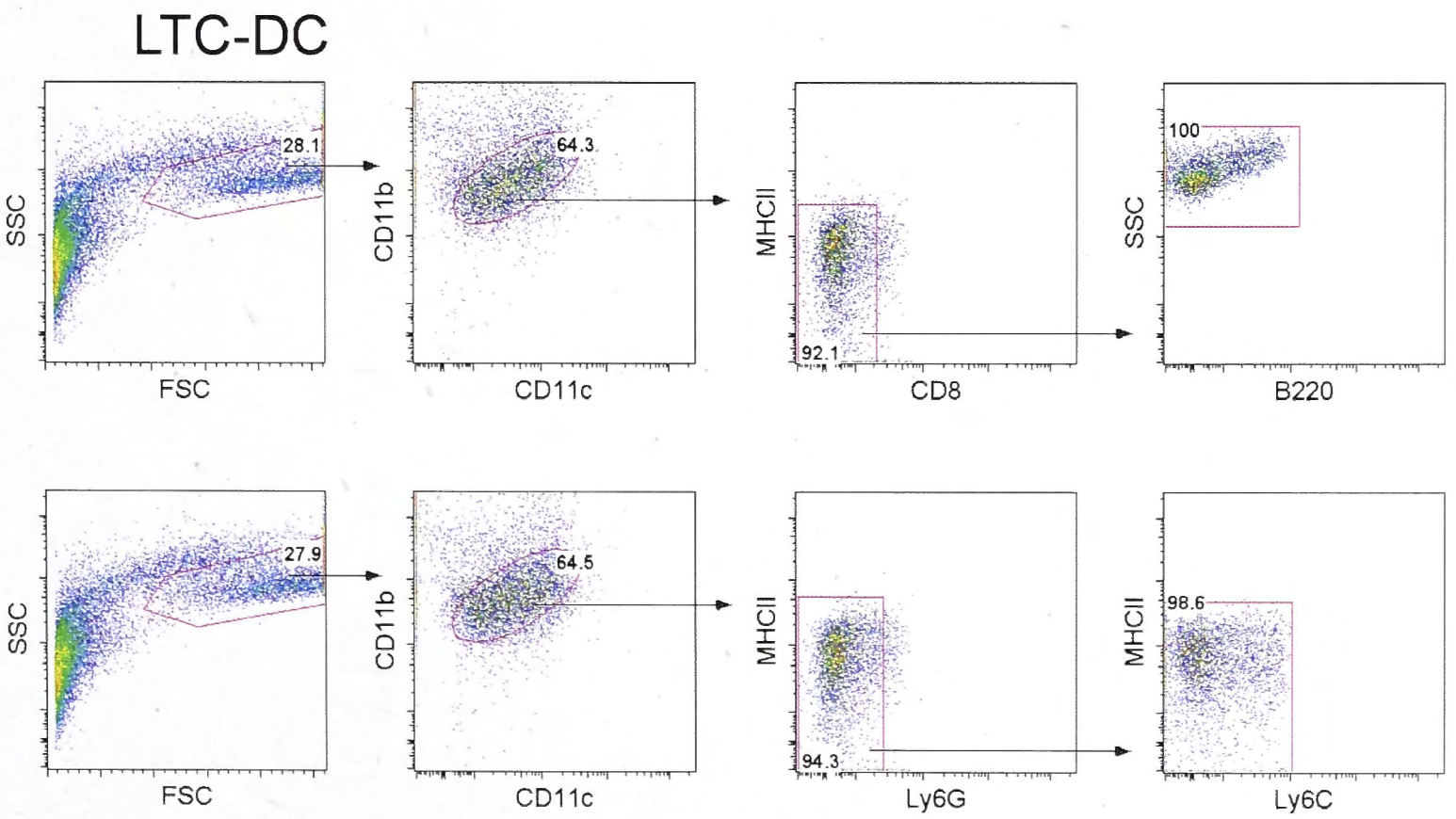
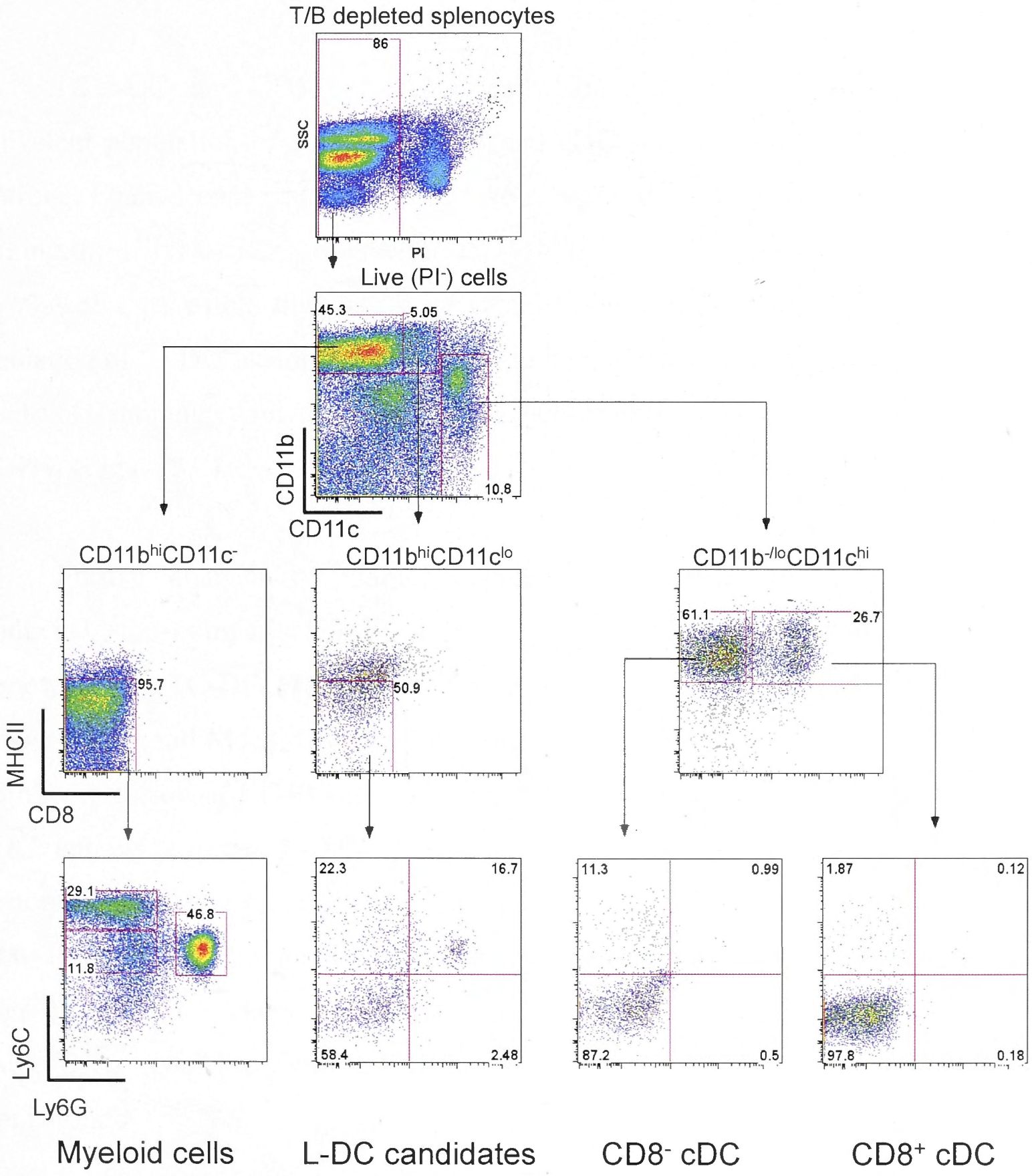


**Figure 3.2 Identification of the *in vivo* equivalent subset of LTC-DC.**

Splenocytes were prepared from C57BL/6J mice. Red blood cells were lysed and T and B cells depleted using magnetic bead technology. Cells were stained with antibodies specific for CD11b (M1/70, PE-Cy7), CD11c (N418, APC), CD8 (53-6.7, Alexa 700), MHCII (M5/114.15.2, Biotin), Ly6C (Al-21, FITC) and Ly6G (1A8, PE). Prior to flow cytometric analysis, cells were stained with propidium iodide (PI, 1µg/ml) for gating live ($PI^-$) cells. Cells were gated based on the known phenotype of LTC-DC, as a $CD11b^{hi}CD11c^{lo}MHCII^-CD8^-$ subset and labelled 'L-DC'. Myeloid cells were gated as $CD11b^{hi}CD11c^-MHCII^-CD8^-$ cells. Both L-DC and myeloid cells were further delineated by Ly6C and Ly6G expression. Conventional DC were gated as $CD11b^{+/-}$ $CD11c^{hi}Ly6C^-Ly6G^-$ cells, and further delineated on the basis CD8 and MHCII expression to give $CD8^+$ cDC and $CD8^-$ cDC. Gates were set based on fluorescence minus one controls and numbers in gates represent % specific binding.


T/B depleted splenocytes
86
SSC
PI
Live (PI-) cells
45.3
5.05
10.8
CD11b
CD11c
CD11bhiCD11c-
CD11bhiCD11clo
CD11b-/loCD11chi
95.7
50.9
61.1
26.7
MHCII
CD8
29.1
46.8
11.8
22.3
16.7
58.4
2.48
11.3
0.99
87.2
0.5
1.87
0.12
97.8
0.18
Ly6C
Ly6G
Myeloid cells
L-DC candidates
CD8- cDC
CD8+ cDC

cells, and 95.7% of cells were gated as $CD8^{-}MHCII^{-}$ to eliminate DC, activated macrophages and B cells from the L-DC gating (Figure 3.2). Ly6C and Ly6G staining revealed 3 major subsets amongst myeloid cells. These have been described in the literature as $Ly6C^{hi}Ly6G^{-}$ inflammatory monocytes, $Ly6C^{lo}Ly6G^{-}$ resident monocytes and $Ly6C^{+}Ly6G^{+}$ granulocytes present in blood (Auffray et al., 2009; Fleming et al., 1993; Galli et al., 2011).

LTC-DC are $CD11b^{hi}CD11c^{lo}$ cells. In order to delineate an *in vivo* equivalent population of L-DC, myeloid and cDC subsets were used as biological controls. Fluorescence minus one controls were used to delineate background staining. Initially, L-DC were gated as $CD11b^{hi}CD11c^{lo}$ cells, and a large proportion (50.9%) of cells within this gate were found to be $CD8^{-}MHCII^{-}$ (Figure 3.2). This population of 'L-DC' candidate was found to be heterogeneous with respect to Ly6C and Ly6G staining, with the $Ly6C^{-}Ly6G^{-}$ subset being the largest sub-population (58.4%) (Figure 3.2).

Further analysis of marker expression on gated cell populations was conducted, for comparison with LTC-DC. L-DC candidates display a similar phenotype to LTC-DC (Table 3.1). In contrast with LTC-DC, L-DC candidates express CD24 and Mac3. Conventional DC are distinguished from L-DC and LTC-DC by expression of CD40 and MHCII (Table 3.1). In addition, both LTC-DC and L-DC subsets express F4/80. Both $CD8^{+}$ cDC and $CD8^{-}$ cDC show similar phenotypic profiles, except that $CD8^{-}$ cDC express the myeloid markers CD11b and F4/80 (Table 3.1). The gated $CD11b^{hi}CD11c^{-}MHCII^{-}CD8^{-}$ myeloid cells express a range of myeloid markers including CD11b, CD24, F4/80, Ly6C, Ly6G and Mac3 (Table 3.1), consistent with the heterogeneous nature of the 'L-DC candidate' population.

### 3.2.3 Functional ability of LTC-DC

One clear property of LTC-DC is their ability to cross-prime $CD8^{+}$ T cells (Quah et al., 2004). In this respect, LTC-DC resemble cDC and particularly $CD8^{+}$ cDC (Joffre et al., 2012). To demonstrate their ability to cross-present antigen, LTC-DC were collected from LTC and stained as described in Figure 3.1 for FACS

**Table 3.1 Comparison of marker expression on LTC-DC and *ex vivo* isolated splenic dendritic and myeloid subsets.**

| Marker | LTC-DC | L-DC | $CD8^+$cDC | $CD8^-$cDC | Myeloid cells |
|---|---|---|---|---|---|
| CD11b | +++ | +++ | - | + | +++ |
| CD11c | + | + | +++ | +++ | - |
| CD8 | - | - | + | - | - |
| CD24 | - | ++ | + | + | ++ |
| CD40 | - | - | + | + | - |
| F4/80 | ++ | ++ | +/- | ++ | +++ |
| Ly6C | - | +/- | - | - | +/- |
| Ly6G | - | - | - | - | +/- |
| Mac3 | - | + | - | - | ++ |
| MHCII | - | - | ++ | ++ | - |
| NK1.1 | - | - | - | - | +/- |

Expression of markers was determined by antibody staining and flow cytometry.
+++, 75-100% cell staining; ++, 50-75% cell staining; +, 25-50% cell staining; +/-, 5-25% cell staining; -, < 5% cell staining.

sorting. Isolated LTC-DC were then pulsed with ovalbumin (OVA) as specific antigen or with the control antigen, hen egg lysozyme (HEL), in the presence or absence of lipopolysaccharide (LPS) added as an activator. Antigen-plused LTC-DC were co-cultured with labelled $CD8^+$ T cells. Freshly isolated splenic $CD11c^+$ cells were used as control antigen presenting cells (APC) in the assay. Diluting numbers of antigen-pulsed LTC-DC or control $CD11c^+$ splenic DC, were co-cultured with CFSE-labelled OT-I (TCR-tg; anti-$OVA/K^b$) $CD8^+$ T cells, or OT-II (TCR-tg; anti-$OVA/IA^b$) $CD4^+$ T cells. After 4 days, T cells were collected, stained for CD4 or CD8 as well as TCR-Vα2, and assessed for reduction in CFSE staining as cells proliferated.

LTC-DC demonstrated strong ability to cross-prime $CD8^+$ T cells in comparison with $CD11c^+$ cells, in both the presence and absence of LPS (Figure 3.3). In contrast, LTC-DC showed no ability to present antigen to $CD4^+$ T cells, while splenic $CD11c^+$ DC were strong activators (Figure 3.3). In all cases of T cell activation, responses were antigen specific for OVA, and were significantly greater in the presence of LPS ($p \leq 0.05$, Student's t-test).

### 3.2.4 Identification of L-DC on the basis of function

The cross-priming ability of LTC-DC was used as a further criterion for delineation of the *in vivo* equivalent L-DC subset. Both the $CD11b^{hi}CD11c^{lo}CD8^-MHCII^-$ candidate L-DC and the $CD11b^{-/lo}CD11c^{hi}CD8^{+/-}MHCII^+$ cDC populations were sorted as described in Figure 3.2. Freshly isolated $CD11c^+$ spleen DC were prepared as a positive control. Cells were then pulsed with specific antigen OVA, or control antigen HEL, in the presence and absence of LPS (Figure 3.4). The cDC populations served as controls for cross-priming OT-I (TCR-tg) $CD8^+$ T cells. In line with previous findings, both $CD8^+$ cDC and $CD8^-$ cDC were able to cross-prime $CD8^+$ T cells (Guermonprez et al., 2002). $CD11b^{hi}CD11c^{lo}CD8^-MHCII^-$ L-DC showed weaker ability to cross-prime, although responses were antigen-specific, and stronger in the presence of LPS (Figure 3.4). The low representation of cells in spleen limited the number of DC available for experimentation, but this initial test confirmed the presence of cross-presenting DC within the L-DC candidate subset.

**Figure 3.3 Antigen presenting ability of LTC-DC.**

Non-adherent cells were collected from LTC and stained with antibodies specific for CD11b (M1/70, PE-Cy7), CD11c (N418, APC), MHC-II (25-9-17, Biotin) and CD8 (53-6.7,PE). LTC-DC were sorted as a $CD11b^{hi}CD11c^{lo}MHCII^{-}CD8^{-}$ subset. $CD11c^{+}$ cells freshly isolated from spleen using MACS magnetic bead technology were prepared as a positive control population. Diluting numbers of APC in triplicate were plated and pulsed with antigen (OVA, or HEL as a control) (10μg/ml) overnight, with or without lipopolysaccharide (LPS) (10μg/ml). After pulsing, cells were washed *in situ* prior to addition of $10^5$ CFSE-labelled OT-I (TCR-tg) $CD8^{+}$ T cells or OT-II (TCR-tg) $CD4^{+}$ T cells. These were purified from mouse spleen by depletion of B cells, DC and myeloid cells using Dynabead and MACS magnetic bead protocols. Cells were co-cultured at T:APC ratios of 2:1, 10:1 and 100:1. After 4 days, T cells were collected and stained with antibodies and propidium iodide (PI, 1ug/ml) for gating live ($PI^{-}$) cells. $PI^{-}CD11b^{-}$ $Thy1.2^{+}V\alpha2^{+}$ cells were gated and proliferation assessed in terms of CFSE dilution. The percentage of proliferated T cells was estimated as a measure of APC capacity. Data are shown as the mean ± S.E of 3 replicates. This experiment was performed by Pravin Periasamy (Periasamy and O'Neill, 2013).

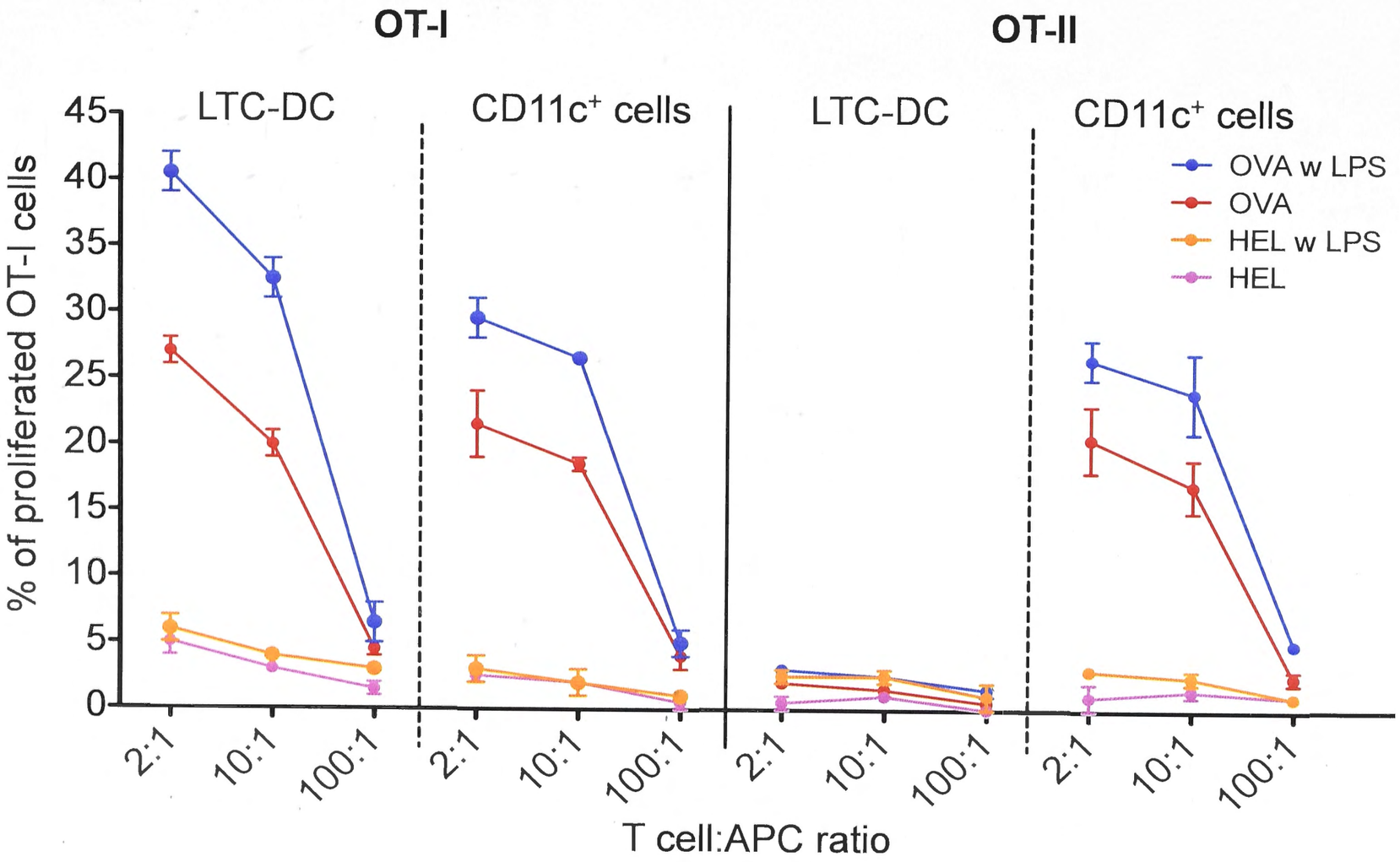
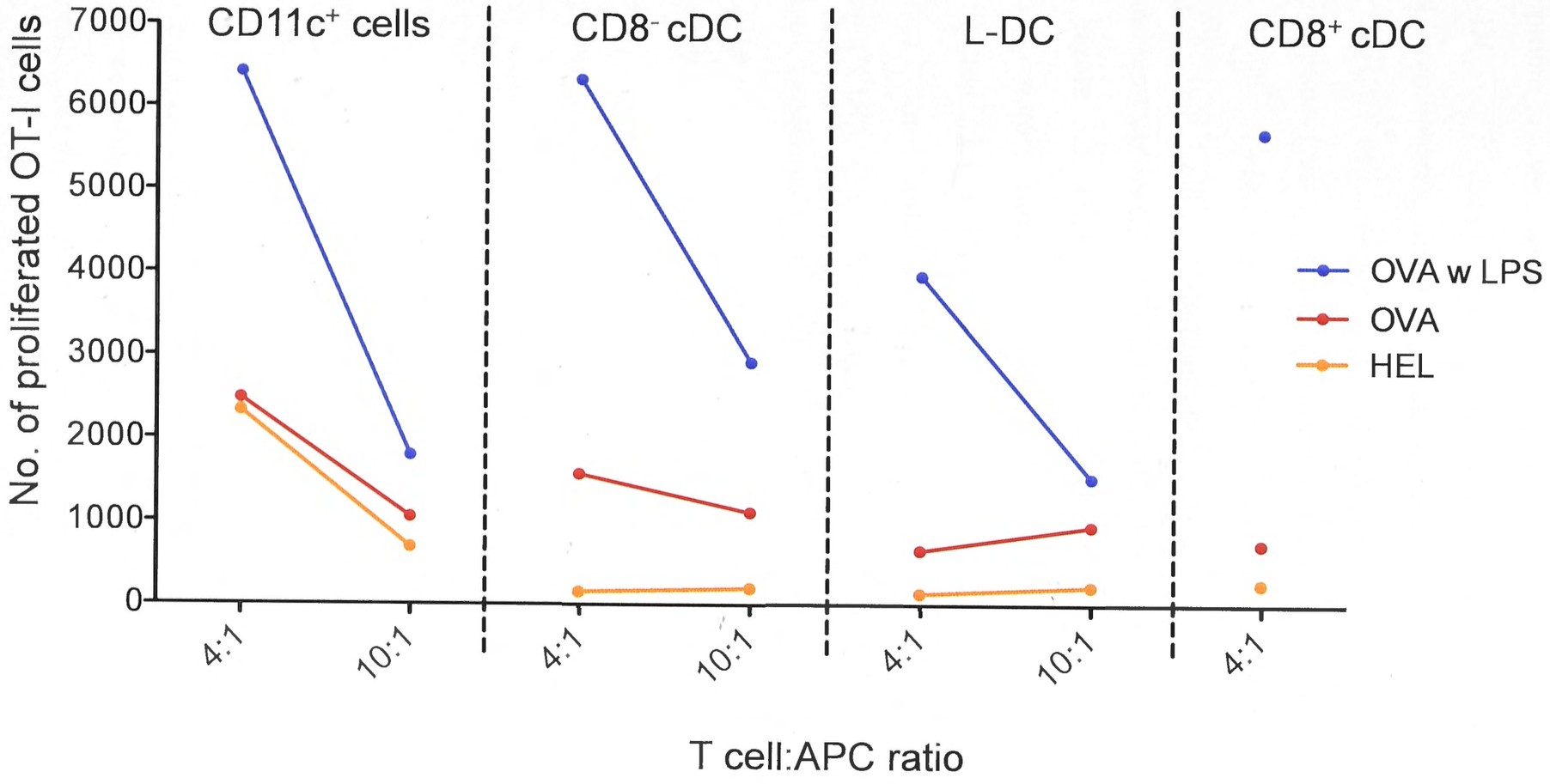

OT-I
OT-II
LTC-DC
CD11c+ cells
LTC-DC
CD11c+ cells
OVA w LPS
OVA
HEL w LPS
HEL
% of proliferated OT-I cells
45
40
35
30
25
20
15
10
5
0
2:1
10:1
100:1
T cell:APC ratio

**Figure 3.4 Cross-priming ability of cDC versus L-DC.**

Splenocytes were prepared and depleted of T and B cells as described in the legend to Figure 3.2. Cells were then stained with antibodies specific for CD11b (M1/70, PE-Cy7), CD11c (N418, APC), MHC-II (25-9-17, Biotin) and CD8 (53-6.7, PE), along with propidium iodide (PI, 1μg/ml) to delineate live ($PI^-$) cells. $CD8^+$ cDC were sorted as $CD11b^-CD11c^{hi}CD8^+MHCII^+$ cells, while $CD8^-$ cDC were sorted as $CD11b^+CD11c^{hi}CD8^-MHCII^+$ cells. L-DC were sorted on the basis of LTC-DC phenotype, as $CD11b^{hi}CD11c^{lo}MHCII^-CD8^-$ cells. Freshly isolated $CD11c^+$ cells were prepared from spleen using MACS magnetic bead technology for use as control APC. Diluting concentrations of APC were plated and pulsed with antigen (OVA, or HEL as control) (10μg/ml) overnight, with or without lipopolysaccharide (LPS) (10μg/ml). After pulsing, cells were washed *in situ* prior to addition of $8x10^4$ CFSE-labelled OT-I (TCR-tg) $CD8^+$ T cells. OT-I cells were purified from spleen as described in Figure 3.3. Where possible, cells were co-cultured in T:APC ratios of 4:1 and 10:1. After 4 days, $CD8^+$ T cells were gated as $PI^-CD11b^-Thy1.2^+V\alpha2^+$ cells. The number of proliferated T cells was assessed in terms of CFSE dilution, and graphed in relation to activating APC.


CD11c+ cells
CD8- cDC
L-DC
CD8+ cDC
No. of proliferated OT-I cells
7000
6000
5000
4000
3000
2000
1000
0
4:1
10:1
4:1
10:1
4:1
10:1
4:1
OVA w LPS
OVA
HEL
T cell:APC ratio

### 3.2.5 Gene profiling to further investigate the L-DC subset

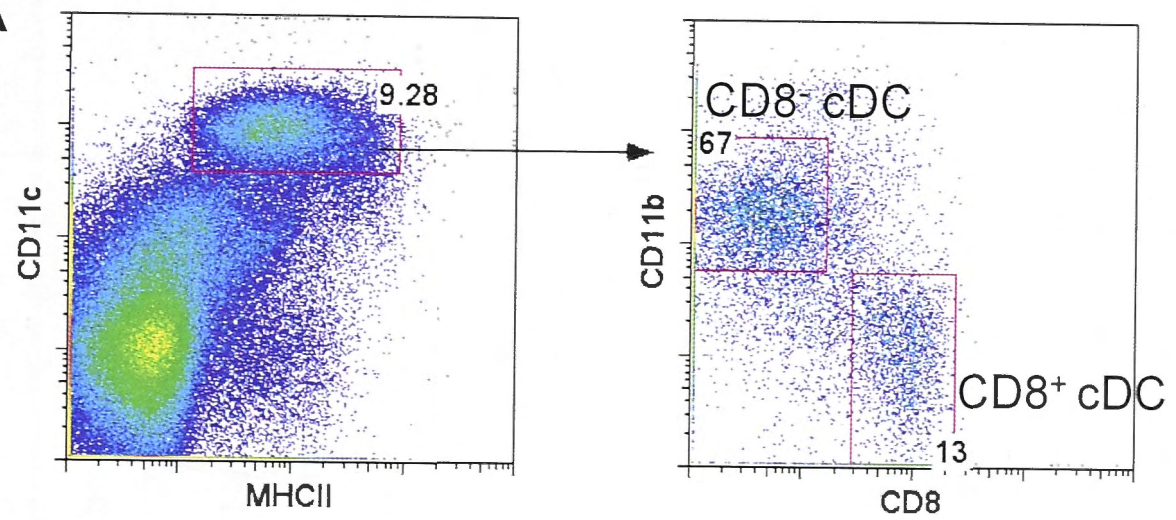
Transcriptome analysis was undertaken to question the relationship between the candidate L-DC subset, the cDC subsets and the myeloid subsets of inflammatory monocytes and granulocytes. In this initial investigation, the broad subset of $CD11b^{hi}CD11c^{lo}MHCII^{-}$ cells, which reputedly contains 'L-DC', was sorted from spleen for transcriptional analysis and comparison with the more clearly defined subsets of $CD8^{+}$ cDC ($CD11b^{-}CD11c^{hi}MHCII^{+}CD8^{+}$), $CD8^{-}$ cDC ($CD11b^{lo}CD11c^{hi}MHCII^{+}CD8^{-}$), inflammatory monocytes ($SSC^{lo}CD11b^{+}CD11c^{-}MHCII^{-}Gr\text{-}1^{-}$) and neutrophils ($SSC^{mid}CD11b^{+}CD11c^{-}MHCII^{-}Gr\text{-}1^{+}$) as delineated in Table 3.2 and Figure 3.5. High purity RNA was extracted from sorted L-DC, granulocytes, inflammatory monocytes and cDC subsets, using the RNeasy minikit as described in Section 2.7.1. RNA of high purity and low degradation was converted to cDNA and then biotin-labelled and hybridised to Murine Gene ST1.0 genechips (Affymetrix: Santa Clara, CA, USA). Due to low cell numbers achieved through sorting, only single genechips were analysed in this initial study aimed at assessing the association between the novel L-DC subset and the myeloid and DC lineages. Scanned images were analysed using Partek (St. Louis, Missouri, USA) to give signal values and p values. Data analysis involved selection of genes up- or down-regulated by $\geq 3$ fold in pairwise comparisons of subsets. Data mining was also used to assess the expression of genes linked to known functions in development, or associated with distinct lineages.

Differences in overall gene expression between the subsets were revealed by Principal Components Analysis (PCA). This showed close grouping between monocytes, $CD8^{+}$ cDC and $CD8^{-}$ cDC in the first principal component, but separation of all but $CD8^{+}$ cDC and $CD8^{-}$ cDC in the second principal component (Figure 3.6). This confirmed similarity between the $CD8^{+}$ cDC and $CD8^{-}$ cDC subsets, and identified the distinctiveness of the granulocyte, inflammatory monocyte and L-DC subsets, both from each other and from cDC.

**Figure 3.5 Cell sorting strategy for subset isolation for gene profiling.**

Splenocytes were harvested from C57BL/6J mice and red blood cells lysed prior to T and B cell depletion. Cells were stained with antibodies specific for CD11b (M1/70, PE-Cy7), CD11c (N418, APC), CD8 (53-6.7, PE), MHCII (M5/114.15.2, FITC) and Gr-1 (RB6-8C5, Biotin). Prior to analysis, cells were stained with propidium iodide (PI, 1μg/ml) for gating live ($PI^-$) cells. (A) cDC were gated as $CD11c^{hi}MHCII^+$ cells and further delineated on the basis of CD8 and CD11b expression. $CD8^+$ cDC were gated as $CD11b^-CD8^+$ cells, while $CD8^-$ cDC were gated as $CD11b^+CD8^-$ cells. (B) The $CD11b^+CD11c^{lo}MHCII^-$ subset was identified and sorted as 'L-DC'. Myeloid cells were gated as $CD11b^+CD11c^-MHCII^-$ cells and further delineated by Gr-1 expression and side scatter (SSC). Monocytes (Mono) were gated as $SSC^{hi}Gr\text{-}1^-$ cells, while inflammatory monocytes (Infl mono) were gated as $SSC^{lo}Gr\text{-}1^-$ cells. Lastly, granulocytes (Granu) were gated as $SSC^{mid}Gr\text{-}1^+$ cells. Gates were set based on isotype controls, and numbers in gates represent % specific binding.

A


9.28
CD11c
MHCII
CD8⁻ cDC
67
CD11b
CD8⁺ cDC
13
CD8

B

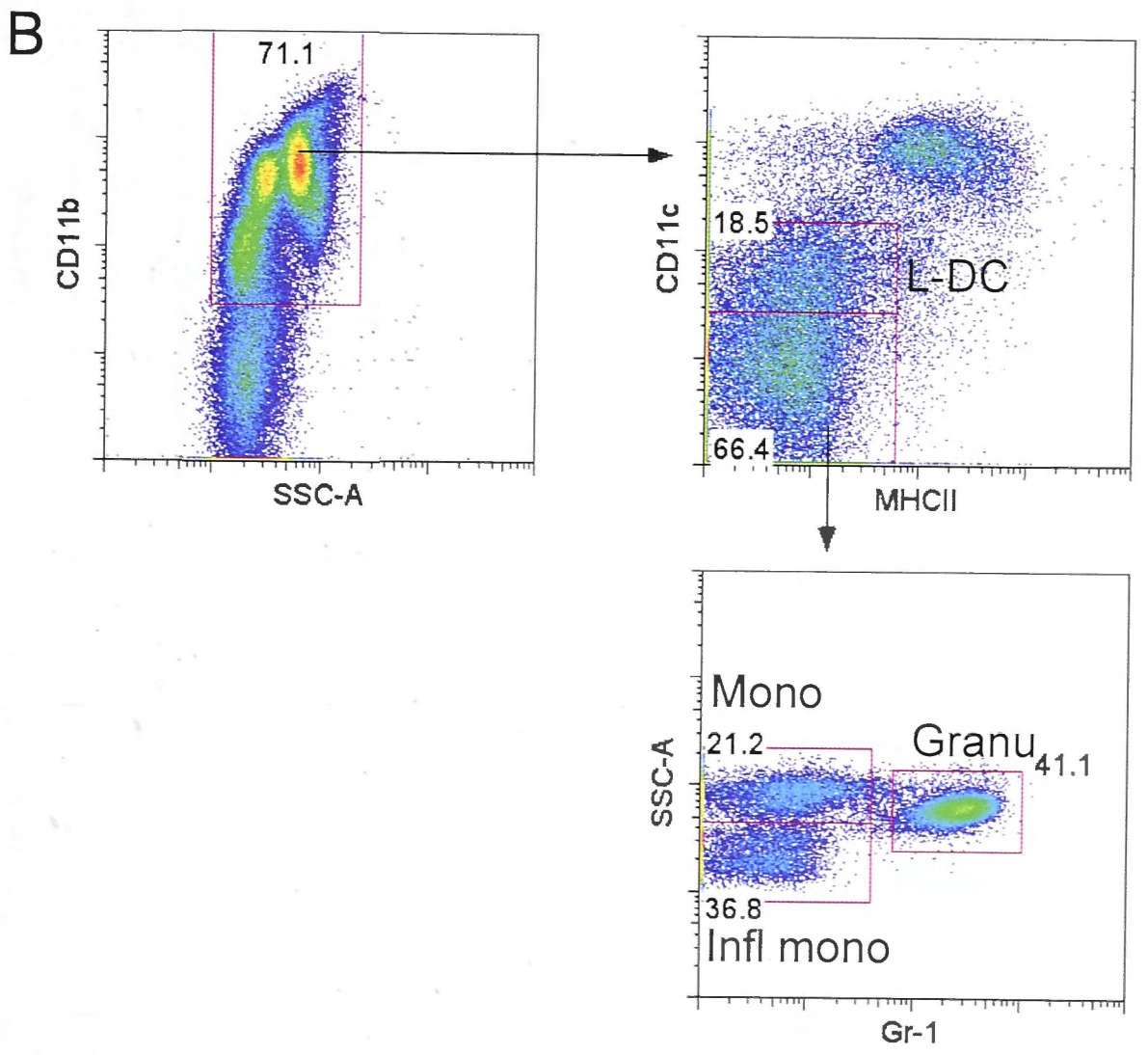
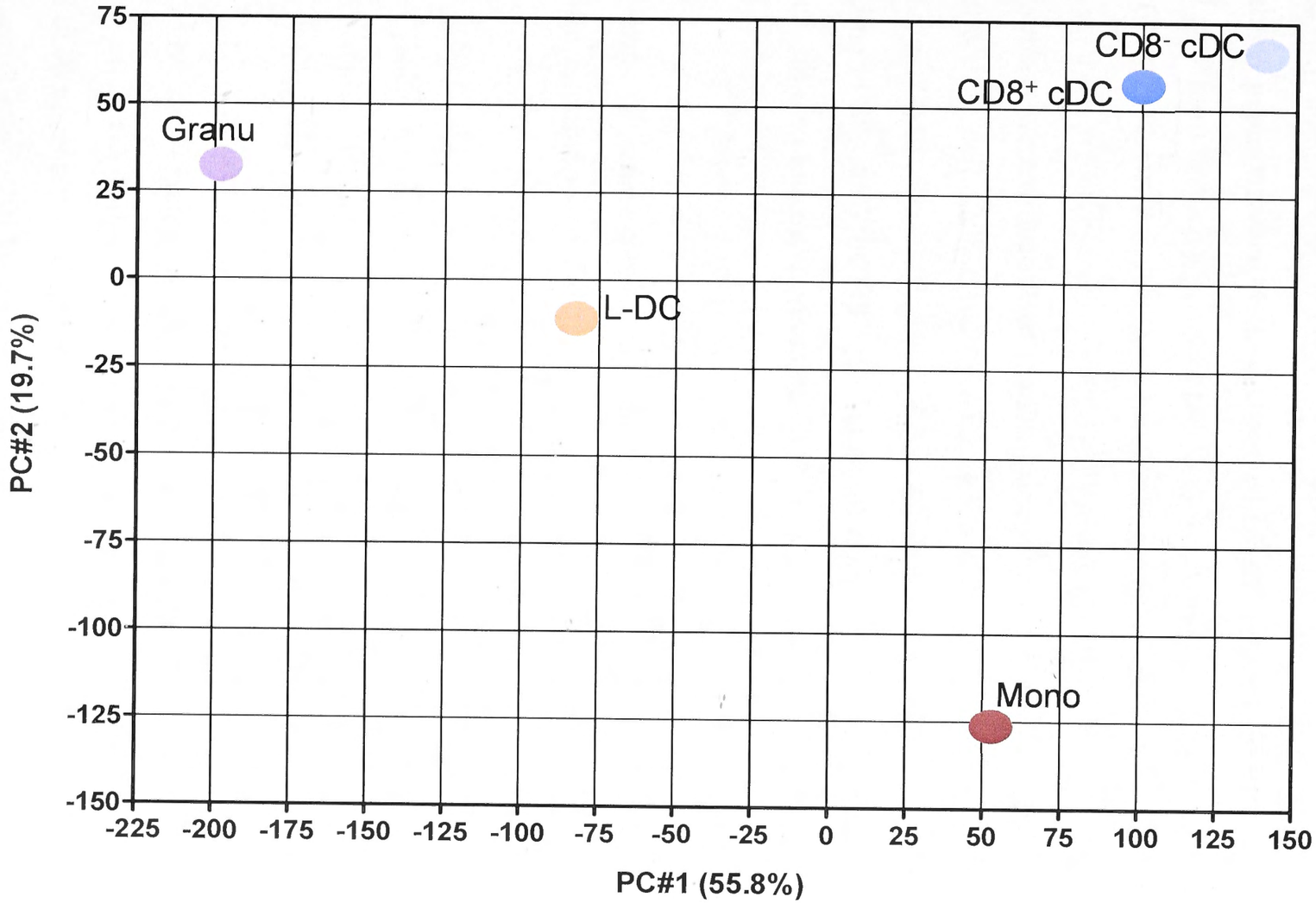

71.1
CD11b
SSC-A
CD11c
18.5
L-DC
66.4
MHCII
Mono
21.2
Granu
41.1
SSC-A
36.8
Infl mono
Gr-1

**Table 3.2 Phenotype of subsets isolated.**

| Subset isolated | Phenotype |
|---|---|
| $CD8^+$ cDC | $CD11b^-CD11c^{hi}MHCII^+CD8^+$ |
| $CD8^-$ cDC | $CD11b^{lo}CD11c^{hi}MHCII^+CD8^-$ |
| L-DC | $FSC^{hi}CD11b^+CD11c^{lo}MHCII^-$ |
| Neutrophils | $FSC^{lo}SSC^{mid}CD11b^+CD11c^-MHCII^-Gr\text{-}1^+$ |
| Inflammatory monocytes | $FSC^{lo}SSC^{lo}CD11b^+CD11c^-MHCII^-Gr\text{-}1^-$ |

**Figure 3.6 Variability in gene expression amongst dendritic and myeloid subsets.**

Transcriptome analysis was performed on sorted murine dendritic and myeloid subsets from spleen as described in Figure 3.5. RNA was extracted and labelled for hybridisation to Murine Gene ST1.0 genechips (Affymetrix). Following data collection, Principal Component Analysis (PCA) was used to determine variability in gene expression between the subsets. Two principal components are shown in the diagram.


75
50
25
0
-25
-50
-75
-100
-125
-150
-225
-200
-175
-150
-125
-100
-75
-50
-25
0
25
50
75
100
125
150
PC#1 (55.8%)
PC#2 (19.7%)
Granu
L-DC
CD8⁻ cDC
CD8⁺ cDC
Mono

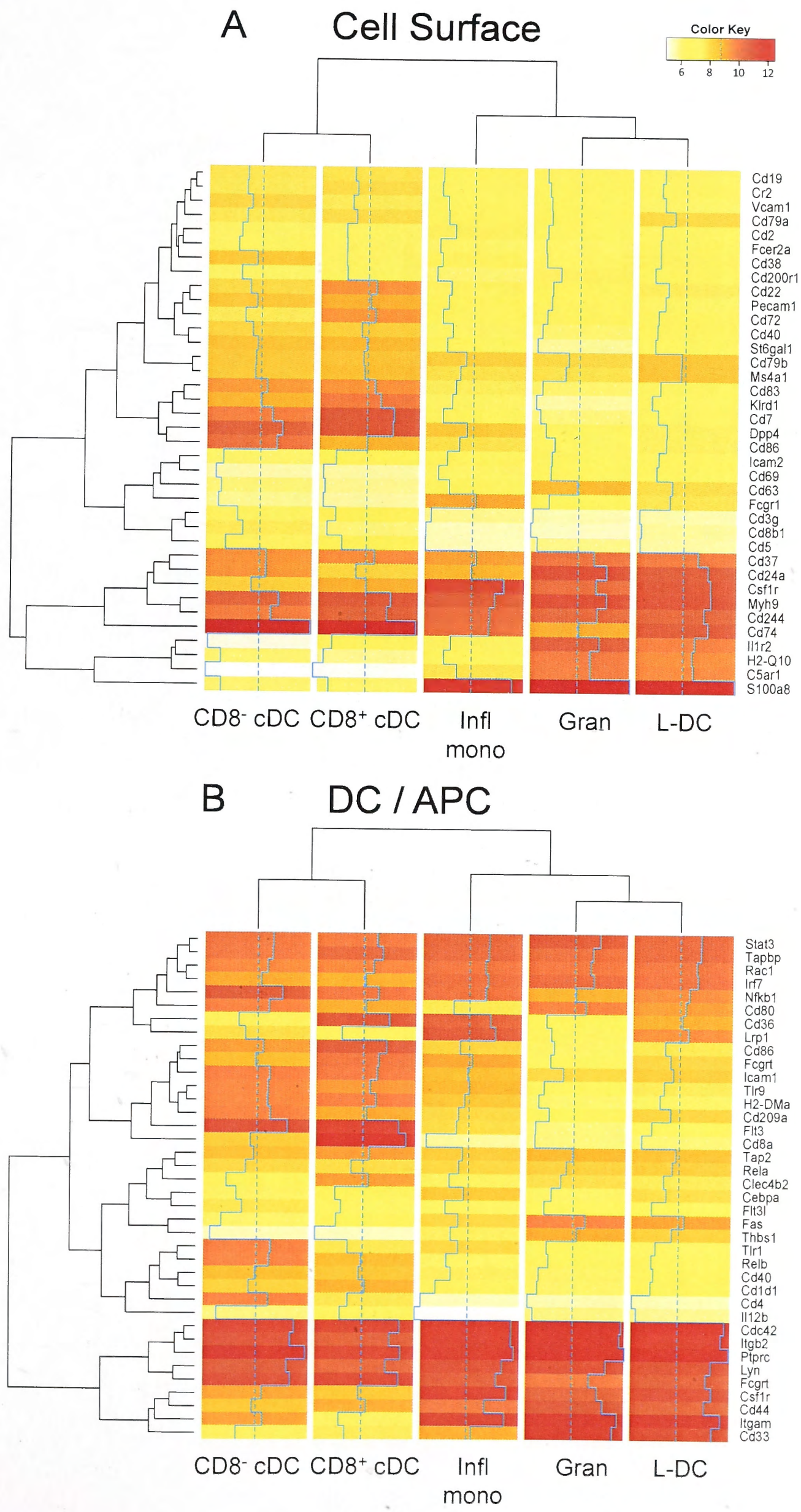
### 3.2.6 Investigation of gene expression

Initially, subsets were investigated by data mining, comparing the expression of known genes in the functional categories of 'DC and APC', 'Chemokines', 'Cell surface markers' and 'Inflammatory cytokines'. Signal values were collected for sets of 84 genes utilised by SABiosciences (Frederick, MD, USA) in their PCR arrays. Data are shown as heatmaps, comparing expression in the five subsets under study for only those genes deemed to be expressed by at least one subset (Figure 3.7). Across all data mining analyses, similarity in gene expression was seen for the $CD8^+$ cDC and $CD8^-$ cDC subsets as shown by dendrograms (Figure 3.7 A-D), consistent with PCA analysis which closely grouped the two cDC subsets (Figure 3.6). Notably these two subsets were reflective of the DC lineage by their common high expression of genes encoding cell surface markers including *Cd74*, *Cd83*, *Cd86*, *Cd40*, *Klrd1*, *Pecam1*, *St6gal1* and *Vcam1* (Figure 3.7 A) (Edwards et al., 2003; Lechmann et al., 2002; Prazma et al., 2007). $CD8^+$ cDC showed high gene expression of *Cd22* and *Cd72* which are also known markers of $CD8^+$ cDC (Edwards et al., 2003; Santos et al., 2008).

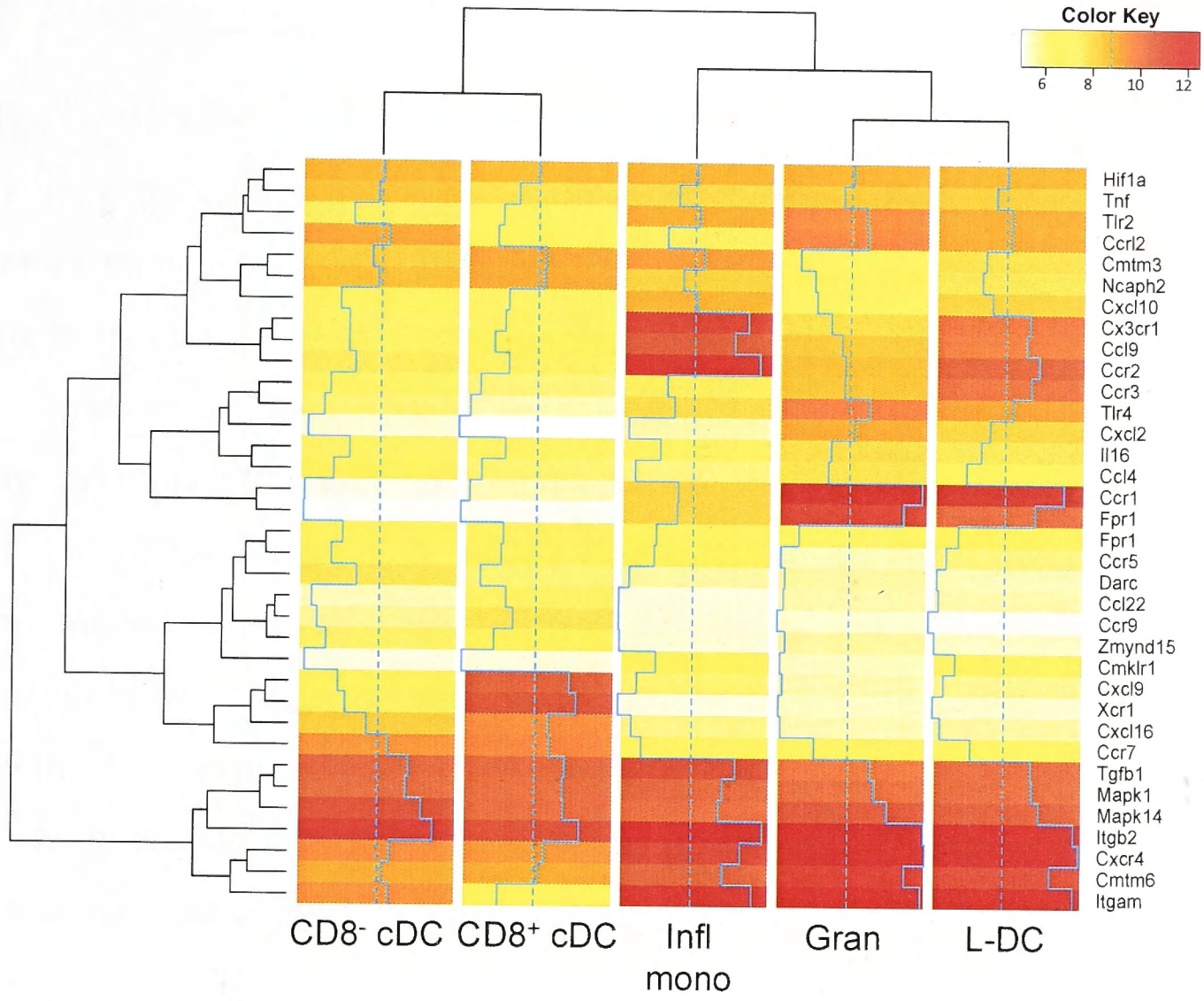
In terms of gene expression related to DC/APC, both cDC subsets showed high specific expression of *Cd1d1*, *Cd86*, *Cd209a*, *Flt3*, *Icam1* and *Relb*, genes which encode known markers of the cDC lineage (Figure 3.7 B) (Caminschi et al., 2001; Edwards et al., 2003; Naik et al., 2006; Vremec et al., 2000). As previously described, $CD8^+$ cDC specifically expressed *Cd8α*, *Cd36*, *Cle4b2* and *Il-12b* (Miller et al., 2012), while $CD8^-$ cDC showed specific expression of *Ter1* and *Cd4* (Miller et al., 2012). In terms of chemokine-related gene expression, both cDC subsets expressed *Ccr7* and *Cxcl16* encoding chemokine and chemokine receptors known to be expressed by cDC (Jang et al., 2006; Riol-Blanco et al., 2005; Sánchez-Sánchez et al., 2006; Van Der Voort et al., 2010). $CD8^+$ cDC were uniquely identified by expression of *Xcr1* as reported previously (Bachem et al., 2010; Kroczek and Henn, 2012; Yamazaki et al., 2013), as well as expression of *Cxcl9*, *Ccr9*, and *Ccl22* for chemokines (Rosenblum et al., 2010). These data and their concordance with descriptions of cDC gene expression in the literature, confirm the efficiency of our sorting and gene profiling protocols. In terms of expression of genes for inflammatory cytokines, it was notable that the cDC subsets were more closely

**Figure 3.7 Gene expression relative to functional capacity**

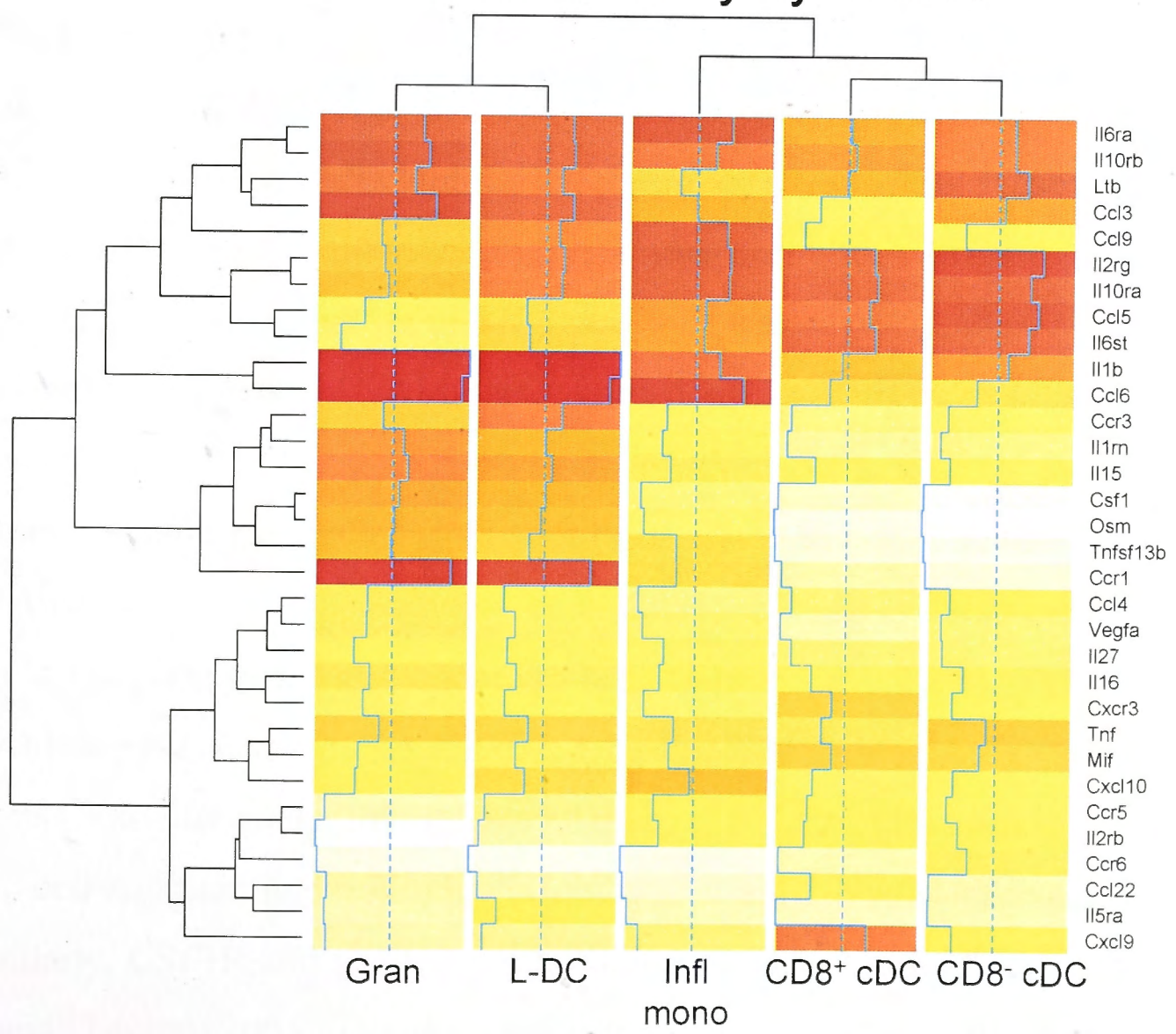
Cell subsets were sorted as described in Figure 3.5 and RNA used to probe Murine Gene 1.0ST genechips (Affymetrix). Data mining was used to extract signal values for sets of genes in functional groupings as used by SAS Bioscience in their PCR arrays. Heat maps were drawn using R/Bioconductor Software, and show those genes which have signal values $\geq 100$ for any one subset. Genes were ordered by level of expression in $CD8^+$ cDC. The line chart (blue) overlaid on heat maps indicates signal intensity changes about the mean (dashed line). Genes were clustered by their level of expression, as shown by row dendrograms. In addition, dendritic and myeloid cell subsets were clustered by gene expression as shown by column dendrograms. Granu: granulocytes; Infl mono: inflammatory monocytes. A) Cell surface markers; B) Dendritic and antigen presenting cells, C) Chemokines and D) Inflammatory cytokines.


A
Cell Surface
Color Key
6
8
10
12
Cd19
Cr2
Vcam1
Cd79a
Cd2
Fcer2a
Cd38
Cd200r1
Cd22
Pecam1
Cd72
Cd40
St6gal1
Cd79b
Ms4a1
Cd83
Klrd1
Cd7
Dpp4
Cd86
Icam2
Cd69
Cd63
Fcgr1
Cd3g
Cd8b1
Cd5
Cd37
Cd24a
Csf1r
Myh9
Cd244
Cd74
Il1r2
H2-Q10
C5ar1
S100a8
CD8- cDC
CD8+ cDC
Infl mono
Gran
L-DC
B
DC / APC
Stat3
Tapbp
Rac1
Irf7
Nfkb1
Cd80
Cd36
Lrp1
Cd86
Fcgrt
Icam1
Tlr9
H2-DMa
Cd209a
Flt3
Cd8a
Tap2
Rela
Clec4b2
Cebpa
Flt3l
Fas
Thbs1
Tlr1
Relb
Cd40
Cd1d1
Cd4
Il12b
Cdc42
Itgb2
Ptprc
Lyn
Fcgrt
Csf1r
Cd44
Itgam
Cd33
CD8- cDC
CD8+ cDC
Infl mono
Gran
L-DC

C Chemokines




D Inflammatory cytokines



related, in terms of clustering, with the inflammatory monocyte subset than with either L-DC or granulocytes. This could be a reflection of the activation status of cells rather than their lineage origin.

The two subsets of granulocytes and 'L-DC candidates' showed high similarity in gene expression across groups of functionally related genes, with inflammatory monocytes as the next related subset. This relationship is shown by dendrograms in Figure 3.7 A-C. Inflammatory monocytes could be identified by specific expression of genes like *Fcgr1*, *CmKlr1*, *Lrp1*, and *Ccr2*, as reported previously by others (Gordon and Taylor, 2005; Grage-Griebenow et al., 2001; Maniecki et al., 2006; Staudt et al., 2013). The L-DC subset showed consistent gene expression in common with granulocytes, suggesting possible overlap or cross contamination between these subsets. In relation to subset contamination, L-DC did show specific high expression of *Ccr3,* and low but specific expression of *IL-5rα*, both of which encode molecules specific to eosinophils (Humbles et al., 2002; Takatsu and Nakajima, 2008). Contamination with these cells is therefore also a possibility.

Genes commonly expressed at high levels across the three myeloid subsets included *IL-6rα*, *Ccl6*, *Tlr2*, *Itgam*, *Itgb2*, *Cxcr4*, *Cmtm6*, *Csfr1*, *Cd44*, *Cd33* and *S100α8* (Figure 3.7 A-D). Myeloid cells expressing IL-6R can respond to IL-6 production associated with inflammatory responses in multiple diseases, and along with TGF-β, induce differentiation of Th17 cells (Grohmann et al., 2001; Kopf et al., 1994; Zhou et al., 2007). ITGAM, also known as CD11b, is a subunit of the heterodimeric integrin MAC-1, expressed by myeloid cells. CD11b mediates the inflammatory response by regulating myeloid cell adhesion and migration to sites of infection (Arnaout et al., 1983; Solovjov et al., 2005). CD11b has also been found to participate in the phagocytosis of apoptotic cells (Chen et al., 2008). The migration of cells involving ITGAM requires ITGB2, a co-factor also commonly expressed by these subsets (Solovjov et al., 2005). Like CD11b, CD44 also plays a role in cell-cell interaction, cell adhesion and migration (Goodison et al., 1999; Marhaba and Zöller, 2004). Similarly, CSF1R and CD33 have been described as myeloid lineage markers (Gordon and Taylor, 2005; Hernández-Caselles et al., 2006). All three subsets expressed high levels of S100α8, a molecule which regulates myeloid cell maturation

and function, and particularly macrophage activation (Donato, 1999; Lagasse and Clerc, 1988; Vogl et al., 2012; Zwadlo et al., 1988). Indeed, all data support the myeloid lineage relationship of L-DC, with a functional relationship rather than a lineage relationship with the cDC subsets.

The L-DC candidate shows a gene profile more in common with inflammatory monocytes and granulocytes than with cDC. Further analysis was therefore aimed at identifying genes specifically upregulated in L-DC and not other subsets, with a view to identification of specific markers and functions which could be used to better delineate the L-DC subset for further characterisation of its development and function as an APC subset in spleen.

### 3.2.7 Identification of genes specifically expressed in the L-DC candidate subset

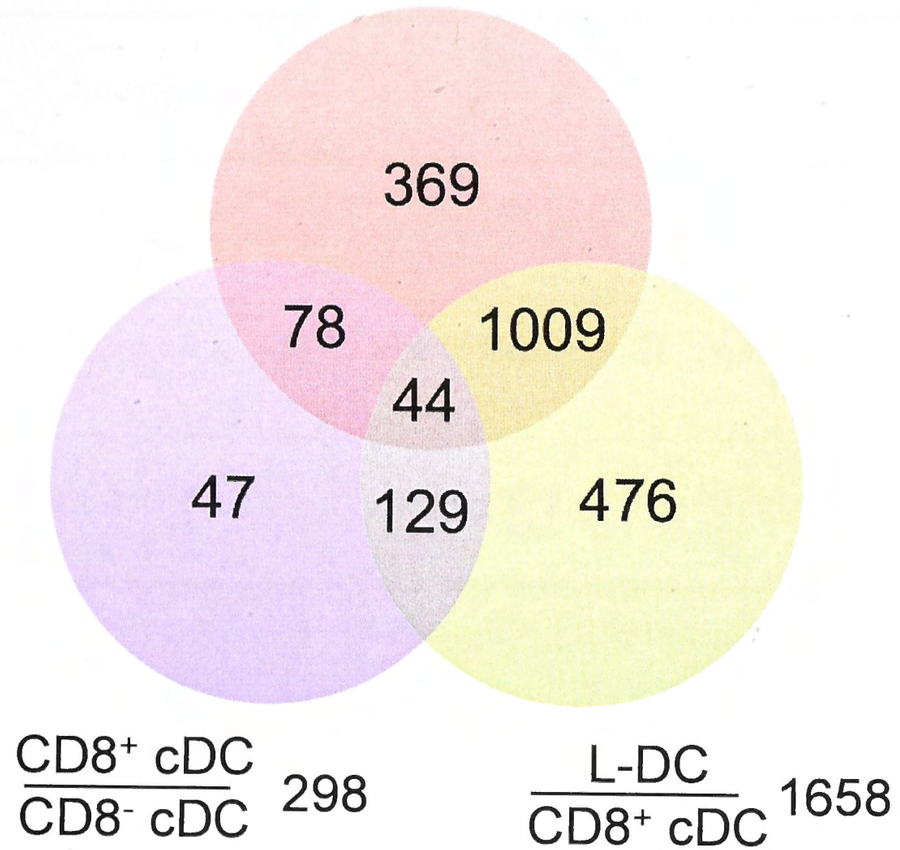
Datasets were extracted containing genes upregulated at least 3 fold in pairwise comparisons of all subsets. The number of genes in each dataset is shown by a Venn diagram which indicates similarity between subsets (Figure 3.8). Amongst the DC subset comparison, lowest variability was found between the $CD8^+$ cDC and $CD8^-$ cDC subsets. Much greater variability was seen between L-DC and each of the cDC subsets. A total of 44 genes were identified which were differentially expressed $\geq$ 3 fold across all 3 subsets (Figure 3.8 A). Amongst the myeloid subset comparisons, inflammatory monocytes and granulocytes were the most distinct, while L-DC and granulocytes were closely related (Figure 3.8 B). Only 10 genes were found to be commonly up- or down- regulated $\geq$ 3 fold in all 3 myeloid subsets. These outcomes are also consistent with a close subset relationship as shown by data mining in Figure 3.7.

Genes which were upregulated in one but not two other subsets were then identified. In the comparison of L-DC with the two cDC subsets, upregulated genes could be identified for all three subsets (Table 3.3). $CD8^+$ cDC subset were identifiable by specific upregulation of the *Cd8a*, *Clec9a*, and *Cd36* genes reported previously (Belz et al., 2002; Caminschi et al., 2008) (Table 3.3). Expression of CLEC9a is restricted to $CD8^+$ cDC and pDC, and has been found to play a role in the

**Figure 3.8 Numbers of differentially expressed genes.**

Diagrams show dataset size for genes upregulated ≥ 3 fold in one of two subsets assessed in pairwise comparison. Granu: Granulocytes; Infl mono: Inflammatory monocytes.

A


L-DC / CD8- cDC 1460
369
78
1009
44
47
129
476
CD8+ cDC / CD8- cDC 298
L-DC / CD8+ cDC 1658

B

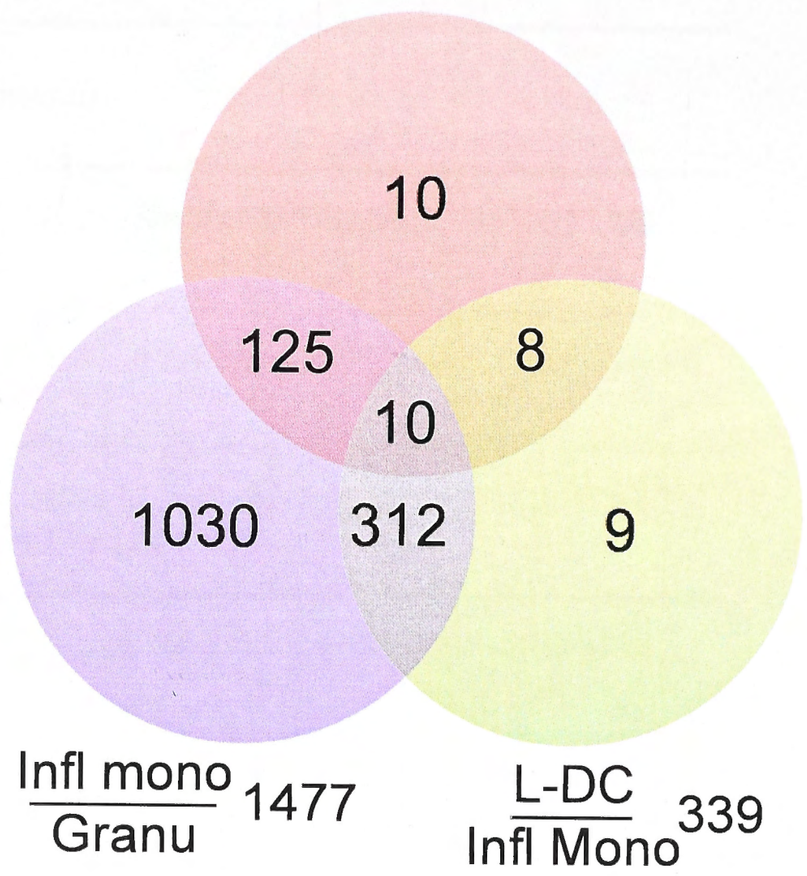

L-DC / Granu 153
10
125
8
10
1030
312
9
Infl mono / Granu 1477
L-DC / Infl Mono 339

**Table 3.3 Genes upregulated in only one of three DC subsets.**

| Subset | Genes upregulated [+] |
|---|---|
| $CD8^+$ cDC | *Cd8α*, *Xcr1*, *Clec9a*, *Cd36*, *Tlr11*, *Tlr3*, *Serpinb9*, *Fndc7*, *Serpinb6b*, *Plekha5*, D330017320, *Apol7c* |
| $CD8^-$ cDC | *Dscam*, 9530009G21, *Ptpn3*, *Ube2e2*, *Stk32c*, *Cd4*, *Centd1*, *Spns3*, *H-2Eb2*, *Sash1*, 1300002K09, *Ctnnd2*, *IL21r* |
| L-DC | *Igsf2*, *Itgam*, *Sirpβ*, *Siglec e*, *Mmp9*, 4732429D16, *Slc2a3*, *Pygl*, *Rtp4*, *Pram1*, 1810033B17, *Ifitm1*, *Cass4*, *Emb*, *Bst1* |

[+] Genes were identified which showed ≥ 3 fold upregulation in signal value in one subset but not the other two, assessed in pairwise comparison.

uptake of apoptotic cells via binding to the exposed actin filament of damaged cells (Caminschi et al., 2008; Zhang et al., 2012). Similarly, CD36 expression has been associated with $CD8^+$ cDC, and participates in the uptake of apoptotic cells (Albert et al., 1998; Belz et al., 2002; Urban et al., 2001). $CD8^+$ cDC were also shown to specifically express *Xcr1*, and XCR1 binds to XCL1 secreted by T cells. XCR1 aids the migration of DC to T cell areas within the spleen (Kroczek and Henn, 2012), which is thought to promote cross-priming of $CD8^+$ T cells and the development of a cytotoxic T cell response (Dorner et al., 2009). Both *Serpinb9* and *Serpinb6b* were also expressed uniquely by $CD8^+$ cDC. These genes encode molecules belonging to the serine protease inhibitor family which participates in many cellular processes including apoptosis and complement fixation. A recent study showed that DC express both SERPINB9 and SERPINB6b, which confer protection against cell death from newly activated cytotoxic T lymphocytes (Bots et al., 2007). *Tlr3* and *Tlr11* gene expression was upregulated in $CD8^+$ cDC (Table 3.3). TLR3 recognises double-stranded RNA, while TLR11 recognises bacteria flagellin (Bauer and Hartmann, 2004), consistent with the ability of $CD8^+$ cDC to act as APC for viruses and bacteria for induction of cytotoxic T lymphocyte responses (Bauer and Hartmann, 2004). Several other genes upregulated in $CD8^+$ cDC included *Fndc7*, *Plekha5* and *Apol7c* which have not previously been associated with this subset.

Known genes upregulated by $CD8^-$ cDC included *Dscam*, *Cd4* and *H-2Eb2*, confirming the identify and purity of the sorted population (Miller et al., 2012). In addition, *Il-21r* gene expression was upregulated in $CD8^-$ cDC. IL-21 has been reported to induce apoptosis amongst cDC via STAT3 and BIM signalling (Wan et al., 2013). Indeed, signalling through the IL-21 receptor could provide a mechanism for regulating cDC numbers. $CD8^-$ cDC also showed high expression of *Sash1* which encodes a signal adaptor protein that participates in cell adhesion and TLR4 signalling (Dauphinee et al., 2013; Martini et al., 2011). While several other known genes were found to be uniquely expressed by $CD8^-$ cDC, none has previously been reported as involved in $CD8^-$ cDC function.

Fifteen genes were shown to be specifically upregulated in L-DC over the cDC subsets. These included *Igsf2* encoding a known marker of human dermal DC which regulates T cell activity (Bagot et al., 1997; Bouloc et al., 2000a). L-DC also

expressed *Sirpβ* which is also expressed by $CD8^-$ cDC, albeit in lower level, but not by $CD8^+$ cDC (Lahoud et al., 2006). Similarly, L-DC specifically expressed *Itgam* which encodes a known marker of $CD8^-$ cDC and myeloid cells (Arnaout et al., 1983; Solovjov et al., 2005). Higher levels of *Siglec e* gene expression were also observed for L-DC in comparison with the cDC subsets. The SIGLEC E molecule on DC has been found to play an inhibitory role in the activation of $CD4^+$ and $CD8^+$ T cells. Antigen taken up via SIGLEC E is rapidly internalised within DC and transported to early endosomes and lysosomes. Presentation of antigen to T cells is diminished (Bax et al., 2010; Boyd et al., 2009; Zhang et al., 2004), leading to limited $CD4^+$ and $CD8^+$ T cell proliferation (Bax et al., 2010; Boyd et al., 2009; Zhang et al., 2004). L-DC also expressed high levels of *Ifitm1*, known to be expressed by DC by 24 hours after their activation (Jin et al., 2010; Yang et al., 2007). IFITM1 displays anti-proliferative activity towards interferon-γ, so regulating the number of T cells (Yang et al., 2007). Lastly, *Pram1* which encodes an adaptor protein involved in T cell receptor-mediated signalling, was also upregulated in L-DC compared with the cDC subsets (Clemens et al., 2004; Moog-Lutz et al., 2001). None of the other L-DC expressed genes listed in Table 3.3 has previously been reported as expressed by DC or APC. Overall, genes upregulated in L-DC reflect functions related to DC, as well as a role for L-DC in the regulation of T effector cell functions.

Further analysis was aimed at identifying genes upregulated in either of L-DC, inflammatory monocytes or granulocytes. However, only inflammatory monocytes showed upregulated genes (Table 3.4). Such a result can be interpreted if granulocytes are a subset of sorted L-DC, and L-DC also contain a subset of inflammatory monocytes. This result confirms our original premise that the L-DC candidate subset is quite heterogeneous, and only partially characterised by the phenotype used here to sort this subset. The close relationship between L-DC and granulocytes, and then with inflammatory monocytes, was identified in initial data mining experiments (Figure 3.7).

Genes specifically upregulated in inflammatory monocytes, included *F10* and *F13a1* which encode coagulation factors (Muszbek et al., 1996; Uprichard and Perry, 2002) (Table 3.4). The F13A1 molecule is also an alternative activation marker

**Table 3.4 Genes upregulated in only one of three myeloid subsets.**

| Subset | Genes upregulated [+] |
|---|---|
| Inflammatory monocytes | *F10*, EG640268, *F13a1*, *Rftn1*, *Cyp27a1*, *Ehd4*, *Scpep1*, *Fyn*, *Cyp2ab1*, *Zfyve9* |
| Granulocytes | nil |
| L-DC | nil |

[+] Genes were identified which showed ≥ 3 fold upregulation in signal value in one subset but not the other two assessed in pairwise comparison.

expressed by macrophages (Martinez et al., 2006). Inflammatory monocytes showed upregulation of *Cyp27a1* and *Cyp2ab1* which encode cytochrome P450 oxidase (Nelson et al., 2004). CYP27A1 is expressed by macrophages and generates 27-hydroxycholesterol which inhibits production of inflammatory factors (Quinn et al., 2005). Lastly, *Fyn* and *Zfyve* which encode signalling molecules were also upregulated in inflammatory monocytes. FYN is involved in the integrin signalling pathway which activates RAS (Wary et al., 1998), and participates in a wide range of cellular pathways including T and B cell receptor signalling, and cytokine receptor signalling (Godfrey et al., 2000; Osterhout et al., 1999; Van Oers et al., 1996). *Zfyve9* encodes an anchor protein involved in TGF-β signalling (Lin et al., 2004). Overall, genes specifically upregulated in inflammatory monocytes, reflect the monocyte/macrophage lineage.

Further investigation of the relationship between L-DC and either inflammatory monocytes or granulocytes, involved a search for genes upregulated in expression between 2 subsets only (Table 3.5). Granulocytes showed no specific gene expression over L-DC, consistent with the interpretation that granulocytes are a subset of the L-DC candidate. In contrast, L-DC showed 144 genes upregulated over granulocytes. A subset of these is shown in Table 3.5. L-DC showed 265 genes upregulated $\geq 3$ fold over inflammatory monocytes, while inflammatory monocytes showed 49 genes upregulated over L-DC. A subset of the most highly upregulated genes is shown in Table 3.5.

Genes upregulated in L-DC over granulocytes reflect myeloid cells in terms of lineage, but DC in terms of functions. For example, *Adamdec1* encodes a myeloid DC marker which becomes upregulated during CD40- or LPS-induced maturation of DC (Fritsche et al., 2003). Another gene of interest is *Cd300e*, known to be expressed by monocytes and myeloid DC (Brckalo et al., 2010; Clark et al., 2009). Upregulation of CD300E in myeloid DC acts as a survival signal (Brckalo et al., 2010), and CD300E the increases production of inflammatory cytokines and the upregulation of costimulatory molecules which regulate T cell activation (Brckalo et al., 2010). Genes associated with cell function and upregulated in L-DC over granulocytes included *Ace*, *Plxnb2* and *Zfyve9*. ACE, also known as CD143, is highly upregulated in monocytes and myeloid DC (Danilov et al., 2003), and

**Table 3.5 Genes upregulated between L-DC and Inflammatory monocytes or Granulocytes.**

| Subset | No. | Genes upregulated[+] |
|---|---|---|
| L-DC | 144 | *Ace*, LOC100047162, *Plxnb2*, *Cd300e*, *Zfyve9*, *Adamdec1*, EG434025 |
| Granulocytes | 0 | nil |
| | | |
| L-DC | 265 | *Igsf2*, *Ens*, *Steap4*, *Arg2*, *Ptgs2*, *Ccr1*, *IL1f9*, *Asprv1*, *Slc7a11*, *DhrS9* |
| Inflammatory monocytes | 49 | *Ncr1*, *NKg7*, *Gzma*, *Klra8*, *Sestd1*, *Fam129b*, *Vwa5a*, *Vcan*, *Pros1* |

[+] Genes were identified which showed ≥ 3 fold upregulation in signal value in one subset but not the other two assessed in pairwise comparison. Only 10 of the most highly upregulated genes are shown.

participates in the presentation of viral antigens to T cells through hydrolysis of peptides (Danilov et al., 2003). PLXNB2 is upregulated in myeloid DC and pDC (Holl et al., 2012), and its primary function is in cell migration and axon guidance (Holl et al., 2012). A recent knockout study showed that *Plxnb2*$^{-/-}$ cells constitutively express IL-12/IL-23p40, suggesting that PLXNB2 negatively regulates production of IL-12 and IL-23, factors which induce Th1 and Th17 responses, respectively (Holl et al., 2012). Lastly, L-DC were shown to express *Zfyve9* which encodes an anchor protein involved in TGF-β signalling (Derynck and Zhang, 2003; Shi, 2000; Tsukazaki et al., 1998), and can induce either a pro-inflammatory or tolerogenic response under different conditions (Wakefield and Roberts, 2002). All of these genes could be regulators of L-DC function as APC which regulate T cell activation and function.

In the comparison of L-DC with inflammatory monocytes, genes with known DC function including *Igsf2*, *Ptgs2, DhrS9* and *IL-1F9* were found to be upregulated by L-DC (Table 3.3). PTGS2 inhibits T cell and DC function via production of prostaglandin $E_2$ (Harizi et al., 2002; Pockaj et al., 2004; Stolina et al., 2000). This supports the activation of DC but suppresses DC ability to attract naïve and effector T cells (Muthuswamy et al., 2010). In addition, prostaglandin $E_2$ also selectively suppresses Th1 and cytotoxic T lymphocyte-mediated immunity, but promotes Th17 and regulatory T cell responses (Kalinski, 2012). *DhrS9* encodes 3-alpha-hydroxysteroid dehydrogenase which is involved in the biosynthesis of retinoic acid (Chetyrkin et al., 2001; Soref et al., 2001), which participates in immune tolerance by enhancing the induction of TGF-β-dependent regulatory T cells, and suppressing the development of Th17 cells (Xiao et al., 2008). Lastly, IL-1F9 also known as IL-36γ, binds to a receptor on DC and $CD4^+$ T cells and induces the production of proinflammatory cytokines (Vigne et al., 2011) which recruit neutrophils, monocytes, DC and macrophages to sites of infection and induce their activation (Miyajima et al., 1992). Again genes specific to L-DC reflect functions of DC and their interaction with T cells in various ways.

Genes found to be upregulated in inflammatory monocytes over L-DC included *Ncr1*, *Nkg7*, *Gzma* and *Klra8*, which are also expressed by natural killer cells. *Gzma* encodes granzyme A expressed by mature natural killer cells and

cytotoxic T lymphocytes (Hameed et al., 1988; Masson et al., 1986). The expression of these genes supports possible contamination of the inflammatory monocyte subset with natural killer cells. However, *Pros1* expressed by inflammatory monocytes reflects a previously reported function of inflammatory monocytes. It encodes an extracellular matrix proteoglycan, upregulated in inflammatory monocytes, which is a key factor in inflammation by interaction with chemokines and adhesion molecules expressed through inflammatory leukocytes (Hirose et al., 2001; Wight, 2002). It also participates in cell migration and proliferation (Wight, 2002). Gene expression analysis between the 3 myeloid subsets confirms suspected subset contamination, and reaffirms the need to define subsets of the myeloid lineage more fully in order to define the L-DC subset in spleen with precision.

## 3.3 Discussion

On the basis of phenotype and function, a candidate *in vivo* equivalent subset of LTC-DC was identified in spleen. This subset has been named 'L-DC', and represents a $CD11b^{hi}CD11c^{lo}CD8^{-}MHCII^{-}$ subset, containing cells with distinct ability to cross-prime $CD8^{+}$ T cells. Gene profiling studies have confirmed that the antibody staining and sorting strategies used here have accurately identified $CD8^{+}$ cDC and $CD8^{-}$ cDC, since their gene profiles reflect cells described in the published literature. However, in terms of identification of myeloid subsets in spleen, the study has been less successful. While sorted granulocytes and inflammatory monocytes displayed markers of the known myeloid subsets, the sorted inflammatory monocyte subset appears to contain some natural killer cells. The sorted L-DC candidate subset expresses both myeloid and DC markers suggesting it is a heterogeneous population. This subset was also shown to contain granulocytes, as well as a subset of cells present in the inflammatory monocyte population. This evidence emphasises how the ability to fractionate subsets is very much dependent on knowledge of the cell surface phenotype of the different cell types. Despite these limitations, this initial gene profiling experiment has been used to advantage to investigate the lineage relationship and function of L-DC with a view to improving our understanding of this novel subset.

L-DC were identified initially on the basis of phenotypic similarity with LTC-DC. However, phenotype alone did not confirm that the $CD11b^{hi}CD11c^{lo}CD8^{-}MHCII^{-}$ population contained an *in vivo* equivalent of LTC-DC. Functional assays however, verified the presence of cells with ability to present exogenous antigen for T cell activation. As with LTC-DC, gated L-DC demonstrated strong ability to cross-prime $CD8^{+}$ T cells in both the presence and absence of LPS.

In terms of gene profiling of subsets isolated from spleen, PCA analysis confirmed that $CD8^{+}$ cDC and $CD8^{-}$ cDC were closely related but quite distinct from the L-DC candidate subset. Both cDC subsets displayed very similar expression profiles across a list of commonly known genes expressed by DC and APC (Figure 3.7 A-D). Both $CD8^{+}$ cDC and $CD8^{-}$ cDC showed upregulated gene expression of *Flt3*, *Cd86*, *Relb* and *Cd209*. CD209 also known as DC-SIGN is a marker which

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L-DC were identified initially on the basis of phenotypic similarity with LTC-DC. However, phenotype alone did not confirm that the $CD11b^{hi}CD11c^{lo}CD8^{-}MHCII^{-}$ population contained an *in vivo* equivalent of LTC-DC. Functional assays however, verified the presence of cells with ability to present exogenous antigen for T cell activation. As with LTC-DC, gated L-DC demonstrated strong ability to cross-prime $CD8^{+}$ T cells in both the presence and absence of LPS.

In terms of gene profiling of subsets isolated from spleen, PCA analysis confirmed that $CD8^{+}$ cDC and $CD8^{-}$ cDC were closely related but quite distinct from the L-DC candidate subset. Both cDC subsets displayed very similar expression profiles across a list of commonly known genes expressed by DC and APC (Figure 3.7 A-D). Both $CD8^{+}$ cDC and $CD8^{-}$ cDC showed upregulated gene expression of *Flt3*, *Cd86*, *Relb* and *Cd209*. CD209 also known as DC-SIGN is a marker which

identifies DC which are monocyte-derived (Cheong et al., 2010) and is expressed by some human macrophages (Rappocciolo et al., 2006). *Relb* encodes a transcription factor expressed by human DC and monocytes (Platzer et al., 2004) which regulates DC activation via NF-kB signalling (Shih et al., 2012). *Flt3* encodes a receptor for FLT3 ligand, an essential growth factor for DC development from common dendritic progenitors and pre-cDC (Karsunky et al., 2003; Kingston et al., 2009). *Cd86* encodes a marker which is upregulated upon DC activation, and both CD86 and CD80 work as co-receptors on DC for activation of T cells (Caux et al., 1994). These data highlight how the sorted cDC subsets studied here express genes and have properties consistent with the reported literature.

The L-DC gene profile was compared with that of $CD8^+$ cDC and $CD8^-$ cDC in order to assess any lineage or functional relationship with DC and to identify genes specifically upregulated in L-DC. *Sirpβ* and *Itgam*, previously described as expressed by $CD8^-$ cDC and myeloid cells, were also found to be upregulated in L-DC. In addition, *Igsf2* expressed by human dermal DC and Langerhans cells (Bouloc et al., 2000b; Grassi et al., 1998) showed upregulated expression in L-DC. L-DC also showed upregulation of genes related to T cell inhibitory roles. Both *Siglec e* and *Igsf2* have been reported to play a role in T cell inhibition. SIGLEC E restricts the presentation of antigen by DC to $CD4^+$ and $CD8^+$ T cells (Bax et al., 2010; Boyd et al., 2009; Zhang et al., 2004), while IGSF2 inhibits T cell proliferation via IL-10 production (Bouloc et al., 2000a). Expression of these genes could reflect regulatory functions of L-DC for T cells. L-DC showed expression of genes common to the DC lineage, and also genes important in antigen presentation or T cell interaction. L-DC therefore reflect DC and APC in terms of gene expression, as well as function in T cell activation. Further gene expression analysis however showed greater similarity between L-DC and myeloid rather than dendritic type cells.

Data mining for expression of genes related to DC and APC function revealed high similarity between L-DC and the myeloid subsets of granulocytes and inflammatory monocytes. Genes included the DC markers *Adamdec1* and *Cd300e* (Brckalo et al., 2010; Clark et al., 2009; Fritsche et al., 2003). They also included genes associated with inhibition of T cell function including *Plxnb2* and *Zfyve9* (Holl et al., 2012; Liu et al., 2008), and genes related to tolerance induction including

*Ptgs2*, *DhrS9* and *Il-1f9* (Muthuswamy et al., 2010; Vigne et al., 2011; Xiao et al., 2008).

The inflammatory monocyte subset sorted here appears to be contaminated with natural killer cells, evidence by upregulated expression of *Ncr1*, *Nkg7*, *Gzma* and *Klra8* (Hameed et al., 1988; Lee et al., 2001; Lieberman, 2003; Masson et al., 1986; Orr et al., 2009). Natural killer cells are a $CD11b^{hi}CD27^{+}Ly6C^{+/-}NK1.1^{+}$ subset of cells (Bezman et al., 2012; Michel et al., 2012; Ryan et al., 1992), and could be contained within the gated inflammatory monocyte subset of $CD11b^{hi}CD11c^{lo}Gr\text{-}1^{-}MHCII^{-}SSC^{lo}$ cells. A more stringent phenotypic profile involving more antibodies will be needed to define inflammatory monocytes more fully.

In summary, the gene profile of the splenic L-DC candidate defined as a $CD11b^{hi}CD11c^{lo}MHCII^{-}CD8^{-}$ subset bears resemblance with myeloid DC, with antigen presenting cells and with cells capable of activating or inhibiting T cells. While this is consistent with evidence for their antigen presenting function, the L-DC candidate subset appears to represent a heterogeneous population resembling LTC-DC, yet showing characteristics of both dendritic and myeloid cells. It is clearly distinct from the known cDC subsets and has greater similarity with myeloid subsets. Further phenotypic characterisation of the L-DC subset has therefore been conducted in Chapter 4, and further analysis of function has been performed in Chapter 5.

Chapter 4

# Delineation of L-DC in relation to splenic dendritic and myeloid cell subsets

## 4.1 Introduction

Multiple dendritic and myeloid subsets have been described in spleen. While splenic dendritic cell (DC) subsets have been very well defined and characterised in terms of phenotype and function, this is not the case for splenic myeloid subsets. The term 'myeloid' has been used as an umbrella term describing both granulocytes and macrophages/monocytes which descend from a common myeloid progenitor (CMP) in bone marrow (Akashi et al., 2000). Granulocytes comprise neutrophils, eosinophils, basophils and mast cells (Hickey and Kubes, 2009), while monocytes have been classified as distinct resident and inflammatory monocytes largely on the basis of monocyte subsets described in peripheral blood (Auffray et al., 2007). The current classification system for splenic myeloid cells is based on cell surface phenotype. However, full phenotypic profiles are not available for these cells, and a benchmark phenotype is lacking in the field. Most studies have focused on specific subsets and do not compare one subset against another to ensure pure populations, or to gain a comprehensive picture of the relationship between subsets. Accurate identification of splenic myeloid cell types is essential for distinguishing specific subsets and their function, and to make studies in the field comparable.

Monocytes develop from a CMP in bone marrow and traverse into blood as mature cells (Akashi et al., 2000). Monocytes in blood have been categorised as both resident and inflammatory monocytes (Auffray et al., 2007; Geissmann et al., 2003). Resident monocytes have a longer half-life than inflammatory monocytes and remain in blood for a longer period (Gordon and Taylor, 2005). In addition, resident monocytes are also thought to act as precursors of tissue resident macrophages in the steady-state, hence the name 'resident' monocytes. They are identifiable as CC-chemokine receptor 2 $(CCR2)^{-}$ and CX3-chemokine receptor 1 $(CX_3CR1)^{hi}Ly6C^{+}Ly6G^{-}$ cells, while inflammatory monocytes are distinct as $CCR2^{+}CX_3CR1^{lo}Ly6C^{hi}Ly6G^{-}$ cells (Auffray et al., 2007; Geissmann et al., 2003; Nahrendorf et al., 2007; Sunderkötter et al., 2004; Tacke et al., 2007). Unlike resident monocytes, inflammatory monocytes are recruited from blood to sites of inflammation and can act as precursors of TNF/iNOS-producing (Tip) DC and macrophages (Geissmann et al., 2008). Recent adoptive transfer studies (Drutman et al., 2012; Varol et al., 2007; Yrlid et al., 2006) suggested that inflammatory

monocytes were precursors of resident monocytes, although this finding has since been disputed (Alder et al., 2008; Mildner et al., 2007). Inflammatory monocytes have also been described as cells which shuffle between bone marrow and blood (Varol et al., 2007), but these findings have also been debated. Further evidence for lineage distinction between these subsets comes from gene knockdown experiments involving the Kruppel-like factor 4 (*Klf4*) molecule, essential for differentiation of inflammatory monocytes (Alder et al., 2008). Gene knockout induced a drop in the number of inflammatory monocytes but did not affect the number of resident monocytes (Alder et al., 2008). In addition, selective depletion of inflammatory monocytes by knockout of *Ccr2* did not affect the number of resident monocytes (Mildner et al., 2007). A direct lineage relationship between resident and inflammatory monocytes is therefore in doubt, and remains to be investigated further in terms of the lineage origin of each subset. It is important to address this issue in the context of defining the lineage relationship of an L-DC subset in spleen.

Most studies on monocytes have involved blood as a source of cells, so that little is understood about splenic monocytes and their relationship with blood monocytes. The studies of Swirski et al. (2009) demonstrated that splenic monocytes resembled their blood counterpart on the basis of phenotype, morphology and gene expression. Monocytes in blood and spleen were identified as lineage $(Lin)^{lo}$ cells, with a $CD11b^{hi}CD11c^{lo}MHCII^{lo}F4/80^{lo}$ phenotype. This population was further delineated into resident and inflammatory monocytes based on Ly6C expression. In addition, spleen was identified as a reservoir for monocytes, where the latter could be efficiently deployed to sites of inflammation during cardiac arrest (Swirski et al., 2009).

Neutrophils in spleen have previously been delineated on the basis of the phenotype of their blood equivalent as a $CD11b^{+}Ly6C^{+}Ly6G^{+}$ subset (Galli et al., 2011), but have not been extensively characterised in spleen. Similarly, eosinophils have been described in blood, but a splenic subset has not been well characterised. Blood eosinophils are defined as a $CD11b^{+}$Siglec-$F^{+}CCR3^{+}F4/80^{+}$ subset (Voehringer et al., 2007), which participates in defence against parasitic infections and allergy via antibody-dependent cell mediated cytotoxicity (Klion and Nutman,

2004; Masure et al., 2013). The precise phenotype of splenic eosinophils relative to other splenic myeloid subsets is still not yet known.

In the previous chapter, L-DC were broadly defined as a subset of cells in spleen with the $CD11b^{hi}CD11c^{lo}MHCII^{-}CD8^{-}$ phenotype. However, this population was found to be heterogeneous on the basis of gene profiling evidence. While L-DC resemble myeloid DC on the basis of CD11b expression, lack of MHCII expression has raised criticism that L-DC may more resemble splenic monocytes/macrophages than DC. In addition, low expression of CD11c on L-DC has raised further questions in that some myeloid DC and pDC have been described as $CD11c^{lo}$ cells (Asselin-Paturel et al., 2001; Björck, 2001; Nakano et al., 2001).

Cell surface phenotype has been widely accepted as a means to identify and distinguish cell subsets. For this reason, a staining protocol and gating strategy is developed here to more accurately identify an L-DC candidate, and to distinguish it from other myeloid and cDC subsets in spleen. In this chapter, the delineation and identification of splenic DC, monocytes and myeloid subsets is addressed concurrently. The phenotype of splenic neutrophils and eosinophils is also characterised in relation to monocytes and DC.

## 4.2 Results

### 4.2.1 Phenotypic characterisation of myeloid subsets in spleen

The identification of known dendritic and myeloid subsets can be readily achieved based on accumulated flow cytometry data in the literature. In line with the commonly used gating strategy, splenic myeloid subsets can be identified by their distinct Ly6C and Ly6G expression. The myeloid subset was gated as $CD11b^{hi}CD11c^{-}MHCII^{-}$. These were further delineated as inflammatory monocytes, resident monocytes and neutrophils (Figure 4.1). Both inflammatory and resident monocytes express Ly6C, but not Ly6G. Inflammatory monocytes were distinguishable from resident monocytes by higher expression of Ly6C. Neutrophils can be distinguished from monocytes, since they express Ly6G, as well as Ly6C (Figure 4.1). In addition, these three myeloid subsets have distinct side scatter profiles (SSC). All three have low forward scatter ($FSC^{lo}$), but can be distinguished based on SSC, with inflammatory monocytes as $SSC^{lo}$, resident monocytes as $SSC^{hi,}$ and neutrophils as $SSC^{mid}$ (Figure 4.1). Hence staining with antibodies specific for CD11b, CD11c, MHCII, Ly6C and Ly6G can distinguish these three myeloid subsets in spleen as described in the literature.

### 4.2.2 Identification of a candidate L-DC subset in spleen

L-DC resemble myeloid DC based on their $CD11b^{hi}$ expression. To further explore the relationship between L-DC and known myeloid subsets in spleen, T and B cell depleted splenocytes were stained for CD11b, CD11c, MHCII, Ly6C and Ly6G. Initially $CD11b^{hi}CD11c^{lo}MHCII^{-}$ cells were gated as the L-DC containing subset, and then further tested for Ly6C and Ly6G expression. Three distinct populations of cells were observed: $Ly6C^{+}Ly6G^{+}$ (21.3%), $Ly6C^{+}Ly6G^{-}$ (32.2%) and $Ly6C^{-}Ly6G^{-}$ (45.4%) (Figure 4.2). These were termed 'L-DC candidates', and further analyses were designed to assess their potential as 'L-DC'. The FSC and SSC profile of each subset was investigated. Both $Ly6C^{+}Ly6G^{+}$ and $Ly6C^{+}Ly6G^{-}$ L-DC candidates have a $FSC^{hi}SSC^{mid}$ profile, while the $Ly6C^{-}Ly6G^{-}$ subset has dominantly a $FSC^{lo}SSC^{lo}$ profile (Figure 4.2).

**Figure 4.1 Flow cytometric identification of myeloid subsets in murine spleen.**

Spleen cells were prepared by red blood cell lysis prior to T and B cell depletion, and then stained with antibodies specific for CD11b (M1/70, PE-Cy7), CD11c (N418, APC), MHCII (M5/114.15.2, Pacific Blue), Ly6C (Al-21, FITC) and Ly6G (1A8, PE). Prior to analysis, cells were stained with propidium iodide (PI, 1μg/ml) for gating live ($PI^-$) cells. Myeloid cells were gated initially as $CD11b^{hi}CD11c^-MHCII^-$ cells. Further staining with Ly6C and Ly6G revealed 3 populations with distinct FSC and SSC as reported in the literature: inflammatory monocytes (Infl mono), resident monocytes (Resi mono) and neutrophils (Neutro). Data are presented as dot plots. Gates were set based on fluorescence minus one controls, and numbers in gates represent % specific binding.

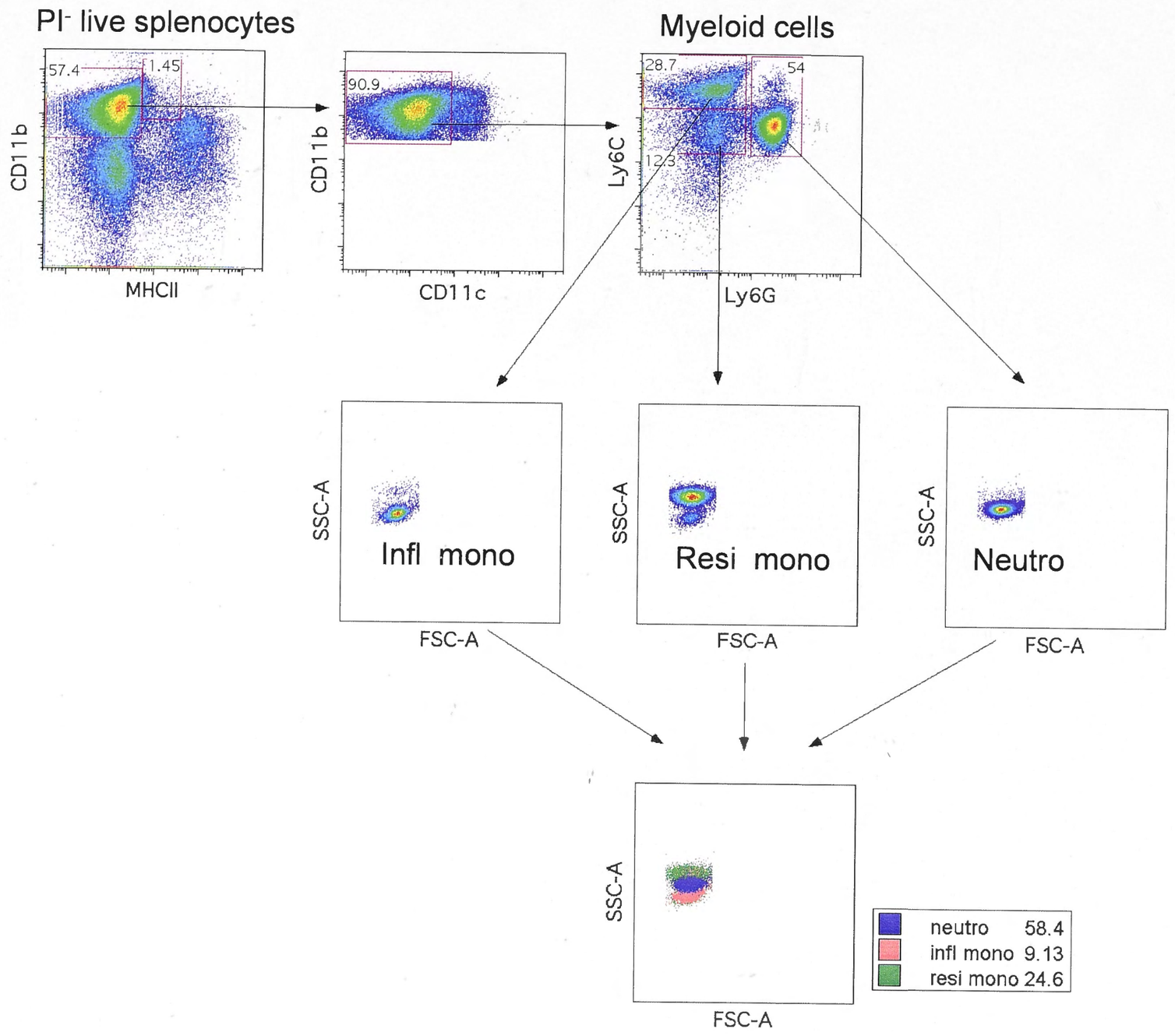
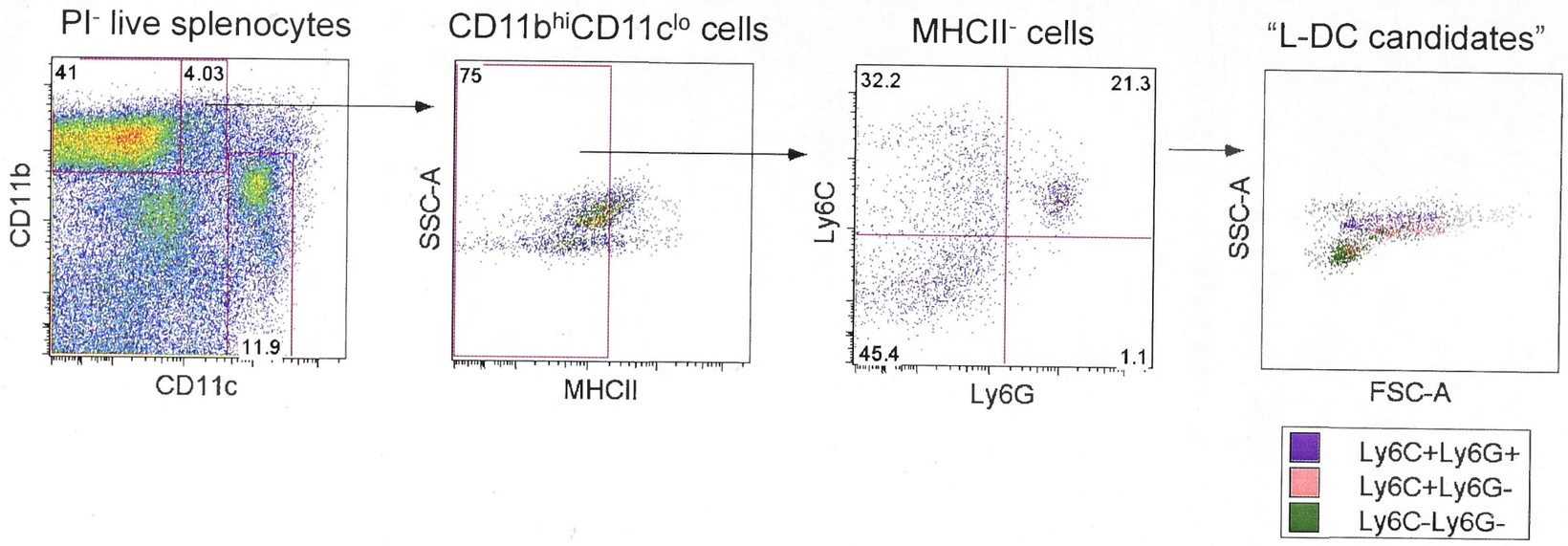

PI- live splenocytes
57.4
1.45
CD11b
MHCII
90.9
CD11b
CD11c
Myeloid cells
28.7
54
12.3
Ly6C
Ly6G
SSC-A
Infl mono
FSC-A
SSC-A
Resi mono
FSC-A
SSC-A
Neutro
FSC-A
SSC-A
FSC-A
neutro 58.4
infl mono 9.13
resi mono 24.6

**Figure 4.2 Initial *ex vivo* identification of L-DC as $CD11b^{hi}CD11c^{lo}MHCII^{-}$ cells.**

Spleen cells were prepared by red blood cell lysis prior to T and B cell depletion, and then stained with antibodies specific for CD11b (M1/70, PE-Cy7), CD11c (N418, APC), MHCII (25-9-17, Biotin), Ly6C (Al-21, FITC) and Ly6G (1A8, PE). Streptavidin (SA)-APC-Cy7 was used as a second stage reagent. Prior to analysis, cells were stained with propidium iodide (PI, 1μg/ml) for gating live ($PI^{-}$) cells. L-DC were initially gated as $CD11b^{hi}CD11c^{lo}MHCII^{-}$ cells. Further staining with Ly6C and Ly6G revealed 3 populations with overlapping FSC and SSC profiles: $Ly6C^{+}Ly6G^{+}$, $Ly6C^{+}Ly6G^{-}$ and $Ly6C^{-}Ly6G^{-}$. Gates were set based on fluorescence minus one controls, and numbers in gates represent % specific binding.


PI- live splenocytes
CD11b
CD11c
41
4.03
11.9
CD11bhiCD11clo cells
SSC-A
MHCII
75
MHCII- cells
Ly6C
Ly6G
32.2
21.3
45.4
1.1
"L-DC candidates"
SSC-A
FSC-A
Ly6C+Ly6G+
Ly6C+Ly6G-
Ly6C-Ly6G-

Since Ly6C and Ly6G expression did not delineate a single subset, additional markers were needed to identify L-DC. To fully delineate the L-DC phenotype, it was also necessary to identify and characterise all known myeloid and DC subsets at the same time. Previously, it was reported that resident monocytes could be distinguished from inflammatory monocytes on the basis of their higher CD43 expression (Sunderkötter et al., 2004). In addition eosinophils, which reside in spleen in the steady-state, are distinguishable from other myeloid cells by expression of Siglec-F (Voehringer et al., 2007). Cells were therefore stained for these markers along with CD11c, CD11b, Ly6C and Ly6G. Common myeloid and DC subsets were gated as in Figure 4.1, and L-DC candidates were gated as in Figure 4.2.

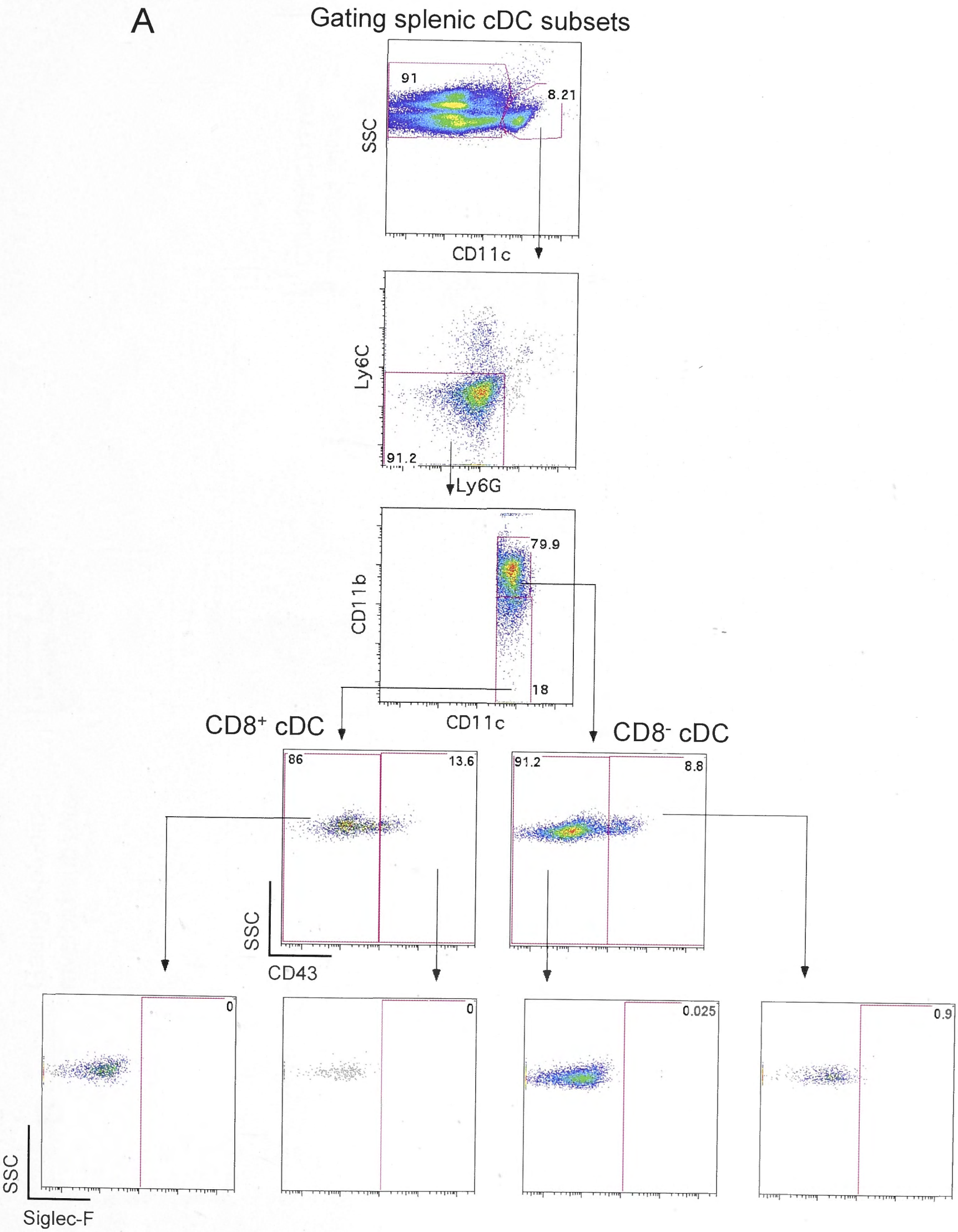
Conventional DC were gated as $CD11c^{hi}Ly6C^{-}Ly6G^{-}$ cells, and $CD8^{+}$ cDC and $CD8^{-}$ cDC were then delineated as the $CD11b^{-}$ and $CD11b^{+}$ subsets, respectively (Figure 4.3 A). While these subsets did not express the Siglec-F marker of eosinophils, a 13.6% subset of $CD8^{+}$ cDC and a 8.8% subset of $CD8^{-}$ cDC were $CD43^{+}$, consistent with published results (Naik et al., 2006). Myeloid cells were gated as $CD11c^{lo/-}$ cells, and further delineated as $CD11b^{hi}CD11c^{lo}$ and $CD11b^{hi}CD11c^{-}$ subsets (Figure 4.3 B). $CD11b^{hi}CD11c^{-}$ cells were further delineated on the basis of Ly6C and Ly6G expression to give inflammatory monocytes, resident monocytes and neutrophils (Figure 4.3 B). Almost 100% of these gated subsets expressed low levels of CD43. Resident monocytes however contained a distinct $CD43^{hi}$ subset (81.8%, Figure 4.3 B). When $CD11b^{hi}CD11c^{lo}$ cells were gated on the basis of Ly6C and Ly6G expression to reveal 3 candidate L-DC subsets, 69.1% of $Ly6C^{-}Ly6G^{-}$, 71% of $Ly6C^{+}Ly6G^{+}$ and 60.6% of $Ly6C^{+}Ly6G^{-}$ cells were $CD43^{+}$. As with resident monocytes, the $Ly6C^{+}Ly6G^{-}$ L-DC candidate also had a $CD43^{hi}$ subset (36.7%, Figure 4.3 B).

Previous studies on eosinophils in the spleen concentrated on their characterisation as a functional cell type and did not focus on their phenotype in relation to other known myeloid subsets. It was therefore important to map out the phenotype of eosinophils in relation to other DC and myeloid subsets under study here. Gated resident monocytes were found to contain a large subset of Siglec-$F^{+}$ cells (87.2%), raising questions about the presence of eosinophils (Figure 4.3 B). In order to confirm the specificity of Siglec-F staining, a back-gating strategy was

**Figure 4.3 Further phenotypic characterisation of splenic dendritic and myeloid subsets.**

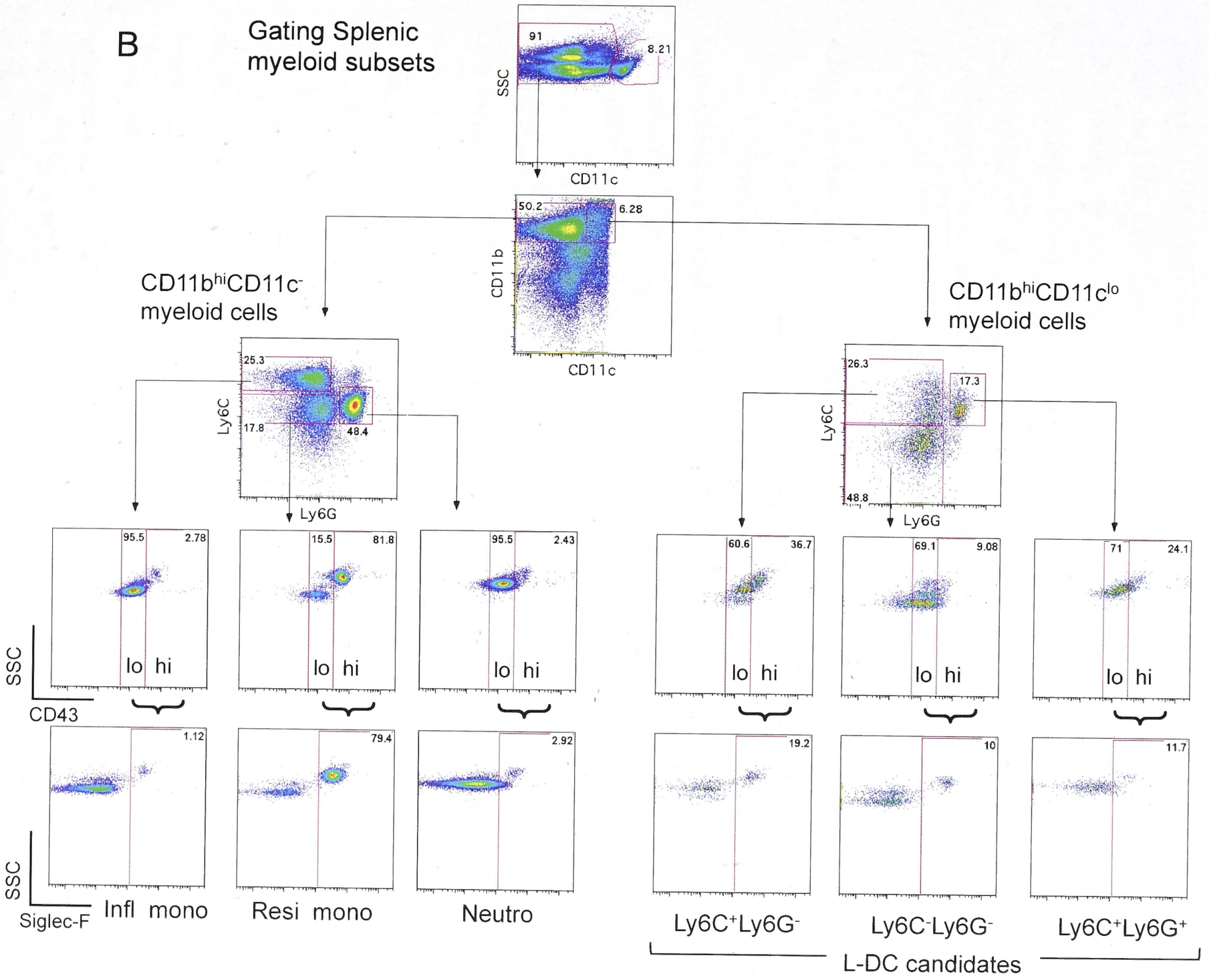
Spleen cells were prepared by red blood cell lysis followed by T and B cell depletion, and then stained with antibodies specific for CD11b (M1/70), CD11c (N418, APC), Ly6C (AL-21, Pacific Blue), Ly6G (1A8, Bio), CD43 (IBII, Alexa488) and Siglec-F (E50-2440, PE), using streptavidin (SA)-APC-Cy7 as a second stage reagent. Prior to flow cytometry, cells were stained with propidium iodide (PI, 1μg/ml) to delineate live ($PI^-$) cells. A) Conventional DC (cDC) were initially gated on the basis of side scatter (SSC) and CD11c expression. $CD8^+$ cDC were gated as $Ly6C^-Ly6G^-$ cells which are also $CD11b^-$, while $CD8^-$ cDC were gated as $Ly6C^-Ly6G^-$ cells which are $CD11b^+$. Expression levels of CD43 and Siglec-F were determined. These are known markers of resident monocytes and eosinophils. B) Myeloid cells and L-DC were initially divided as $CD11c^-$ and $CD11c^{lo}$ subsets, respectively, and further delineated on the basis of CD11b versus CD11c expression. Myeloid cells were gated as $CD11b^{hi}CD11c^-$ cells, and further delineated on the basis of Ly6C and Ly6G expression to reveal neutrophils (Neutro) as $Ly6C^+Ly6G^+$ cells, inflammatory monocytes (Infl mono) as $Ly6C^{hi}Ly6G^-$ cells and resident monocytes (Resi mono) as $Ly6C^+Ly6G^-$ cells, as described in the literature (Geissmann et al., 2003). L-DC were gated as $CD11b^{hi}CD11c^{lo}$ cells, and then further delineated to give 3 candidate subsets differing in Ly6C and Ly6G expression. Expression of CD43 and Siglec-F were determined for all subsets. Gates were set based on fluorescence minus one controls, and numbers in gates represent % specific binding.

A


Gating splenic cDC subsets
91
8.21
SSC
CD11c
Ly6C
91.2
Ly6G
79.9
CD11b
18
CD11c
CD8+ cDC
CD8- cDC
86
13.6
91.2
8.8
SSC
CD43
0
0
0.025
0.9
SSC
Siglec-F

B

Gating Splenic myeloid subsets



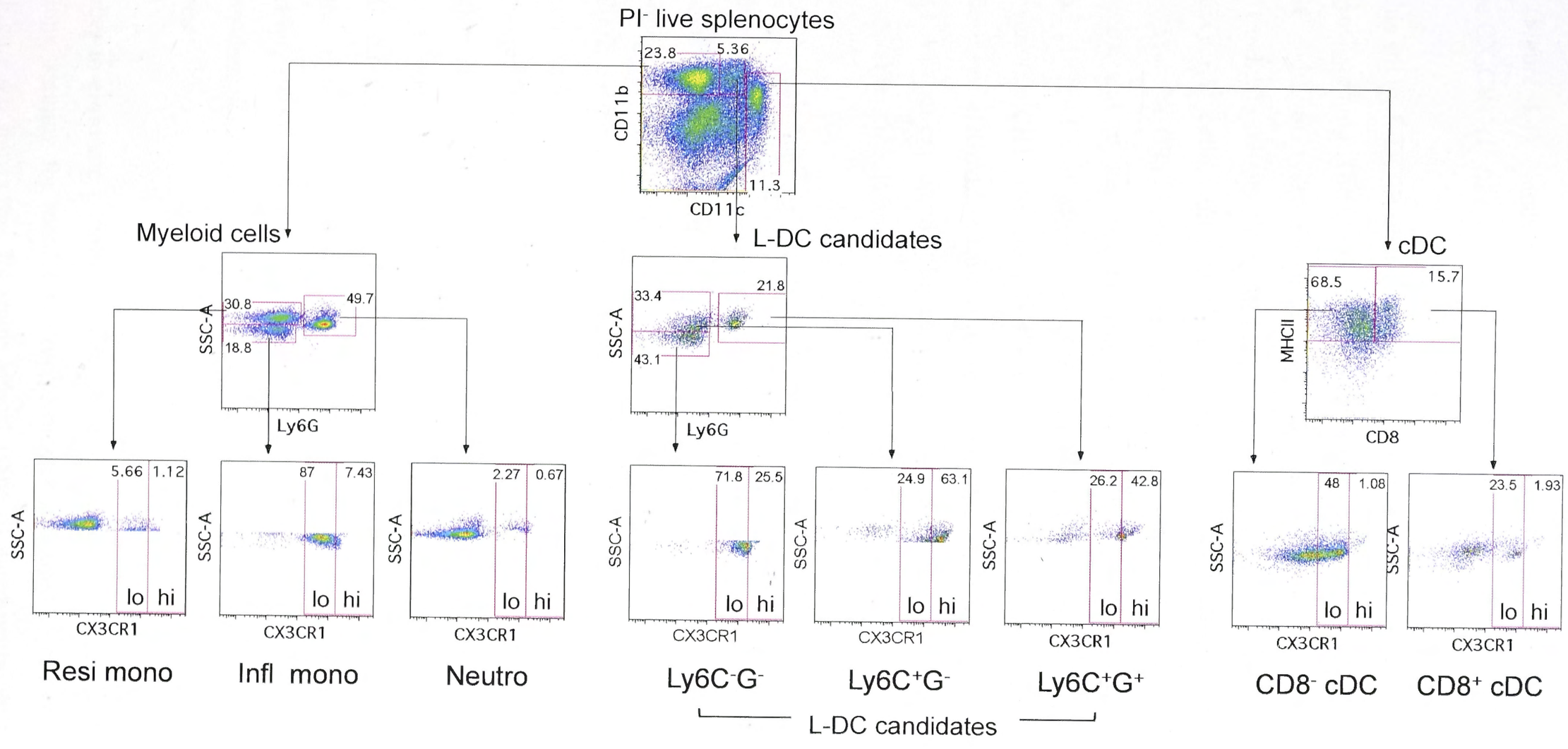
applied to Siglec-$F^+$ cells (Figure A.1). By this method, Siglec-$F^+$ cells were found to be mainly $CD11b^{hi}CD11c^-Ly6C^+Ly6G^-CD43^+$ cells, previously designated as resident monocytes in Figures 4.1 and 4.3. By contrast, the majority of inflammatory monocytes (~99%) and neutrophils (~97%) did not express Siglec-F (Figure 4.3 B). While the $Ly6C^-Ly6G^-$ and $Ly6C^+Ly6G^+$ L-DC candidate subsets showed subsets of cells staining for Siglec-F (Figure 4.3 B), these subsets were small (~10% and ~11.7%, respectively). However, 19.2% of the $Ly6C^+Ly6G^-$ L-DC candidate subset expressed Siglec-F (Figure 4.3 B). This staining pattern indicates possible eosinophil contamination amongst resident monocytes, and amongst the $Ly6C^+Ly6G^-$ L-DC candidate subset.

### 4.2.3 Further distinction of myeloid subsets in spleen

To investigate the subsets further, the phenotype of designated resident monocytes was analysed in detail. $CX_3CR1$ expression has been associated with the differentiation of macrophages and DC, and this lineage has been defined by a macrophage/dendritic progenitor (MDP) (Fogg et al., 2006; Jung et al., 2000; Palframan et al., 2001). Resident monocytes and inflammatory monocytes have been described as $CX_3CR1^{hi}$ and $CX_3CR1^{lo}$ cells, respectively (Auffray et al., 2007; Geissmann et al., 2003). $CX_3CR1$ transgenic mice tagged with green fluorescence protein (GFP) were therefore used to determine if the designated resident monocyte subset of $CD11b^{hi}CD11c^-Ly6C^+Ly6G^-Siglec\text{-}F^+$ cells expressed $CX_3CR1$, which is associated with the macrophage/dendritic lineage. In order to detect staining levels of $CX_3CR1$, two gates were defined based on fluorescence minus one controls, namely, a $CX_3CR1^{lo}$ and a $CX_3CR1^{hi}$ gate, reflecting cells expressing different levels of the marker. Experimental limitations reduced the number of markers which could be studied concurrently, and so Ly6C was not included in the staining mix. Identification of myeloid subsets was therefore based on the SSC profile as described in Figure 4.1, and on Ly6G staining. When designated resident monocytes in spleen were gated as $SSC^{hi}Ly6G^-$ cells, they showed few cells with $CX_3CR1$ staining (6.78%), again raising issues about the identity of cells as resident monocytes (Figure 4.4). Inflammatory monocytes were gated as $SSC^{lo}Ly6G^-$ cells, and consistent with the literature (Geissmann et al., 2003), 87% of cells were $CX_3CR1^{lo}$ with 7.4% as

**Figure 4.4 $CX_3CR1$ expression delineates splenic dendritic and myeloid subsets further.**

Spleens of *Cx3Cr1-GFP* transgenic mice were prepared by RBC lysis and T/B cell depletion. Cells were stained with 2 distinct antibody cocktails. The first included antibodies to CD11b (M1/70, PE-Cy7), CD11c (N418, APC) and Ly6G (1A8, PE), while the second contained antibodies to CD11b (M1/70, Pe-Cy7), CD11c (N418, APC), CD8 (53-6.7, PE) and MHC-II (25-9-17, Biotin). Streptavidin (SA)-APC-Cy7 was used as a second stage reagent. Prior to flow cytometry, cells were stained with propidium iodide (PI, 1μg/ml) to delineate live ($PI^-$) cells. Initial gating on the basis of CD11c and CD11b expression allowed identification of myeloid cells as $CD11b^{hi}CD11c^-$, L-DC candidates as $CD11b^{hi}CD11c^{lo}$, and cDC as a $CD11b^+CD11c^{hi}$ subset. Myeloid cells were further delineated on the basis of SSC and Ly6G expression. Resident monocytes (Resi mono) were gated as $SSC^{hi}Ly6G^-$ cells, inflammatory monocytes (Infl mono) as $SSC^{lo}Ly6G^-$ cells and neutrophils (Neutro) as $SSC^{inter}Ly6G^+$ cells, based on published data (Tan et al., 2011). Similarly, three L-DC candidate subsets were delineated on the basis of Ly6G expression and predicted Ly6C expression. cDC were separated into $CD8^+$ cDC and $CD8^-$ cDC based on MHCII and CD8 expression. $CX_3CR1$ expression was examined across all identified subsets. Gates were set based on fluorescence minus one controls, and numbers in gates represent % specific binding.


PI- live splenocytes
CD11b
CD11c
23.8
5.36
11.3
Myeloid cells
L-DC candidates
cDC
SSC-A
Ly6G
30.8
49.7
18.8
33.4
21.8
43.1
MHCII
CD8
68.5
15.7
5.66
1.12
87
7.43
2.27
0.67
71.8
25.5
24.9
63.1
26.2
42.8
48
1.08
23.5
1.93
lo
hi
CX3CR1
Resi mono
Infl mono
Neutro
Ly6C-G-
Ly6C+G-
Ly6C+G+
CD8- cDC
CD8+ cDC
L-DC candidates

$CX_3CR1^{hi}$ (Figure 4.4). Neutrophils were gated as $SSC^{mid}Ly6G^{+}$ cells, and were shown to be $CX_3CR1^{-}$ (Figure 4.4).

In this study, L-DC candidates were also divided into 3 populations based on SSC and Ly6G staining. The Ly6C expression on the L-DC candidates gated on the basis of SSC and Ly6G was verified in another staining (Figure A.2). Amongst the three L-DC candidate subsets, the $Ly6C^{-}Ly6G^{-}$ subset contained 72% $CX_3CR1^{lo}$ cells with 26% $CX_3CR1^{hi}$ cells, whilst the $Ly6C^{+}Ly6G^{-}$ subset contained 63% $CX_3CR1^{hi}$ cells with 25% $CX_3CR1^{lo}$ cells. The $Ly6C^{+}Ly6G^{+}$ subset contained 43% of $CX_3CR1^{hi}$ cells, and so was also heterogeneous. When $CX_3CR1$ expression was examined on cDC, 48% of gated $CD8^{-}$ cDC were $CX_3CR1^{lo}$, while 24% of gated $CD8^{+}$ cDC were $CX_3CR1^{lo}$ (Figure 4.4). $CX_3CR1$ is a clear marker expressed at low levels on subsets of cDC and inflammatory monocytes. However, here it was found that $CX_3CR1$ was not expressed at high levels on the resident monocyte population as defined in the literature. It was, however, expressed at high levels on subsets of L-DC candidates.

Further attempts were therefore made to distinguish a true resident monocyte subset in spleen. Macrophage-colony stimulating factor receptor (M-CSFR/CD115), was used previously to delineate monocytes/macrophages and other myeloid cells (Dai et al., 2002; Sasmono et al., 2003), and was examined here as a possible marker for further delineation of splenic myeloid and DC subsets. All designated resident monocytes were found to be $CD115^{+}$, while less than 10% of inflammatory monocytes expressed CD115 (Figure 4.5). Neutrophils were $CD115^{-}$, consistent with the literature (Dunay et al., 2010). Amongst the L-DC candidates, 63% of the $Ly6C^{+}Ly6G^{-}$ subset and 45% of the $Ly6C^{+}Ly6G^{+}$ subset expressed CD115. However, the $Ly6C^{-}Ly6G^{-}$ L-DC candidate was clearly negative for CD115 expression (Figure 4.5). In terms of CD115 expression, overlap exists between the designated resident monocytes and the $Ly6C^{+}Ly6G^{+}$ and $Ly6C^{+}Ly6G^{-}$ subsets of L-DC candidates.

In order to eliminate possible contamination of neutrophils within gated L-DC populations, staining for the 7/4 neutrophil marker was used to assess L-DC candidates. Figure 4.6 shows that 7/4 stains almost 100% of gated neutrophils as a

**Figure 4.5 Identification of splenic monocytes on the basis of CD115 expression.**

Splenocytes were prepared by red blood cells lysis and T/B cell depletion and stained with antibodies specific for CD11b (M1/70, PE-Cy7), CD11c (N418, APC), Ly6C (Al-21, FITC), CD115 (AFS98, PE) and Ly6G (1A8, biotin). Streptavidin (SA)-APC-Cy7 was used as a second stage reagent. Prior to analysis, cells were stained with propidium iodide (PI, 1μg/ml) for gating live ($PI^-$) cells. Myeloid subsets of inflammatory monocytes (Infl mono), resident monocytes (Resi mono), neutrophils (Neutro) and L-DC candidates were gated as described in Figure 4.3. CD115 expression levels were determined on gated subsets. Gates were set based on fluorescence minus one controls, and numbers in gates represent % specific binding.

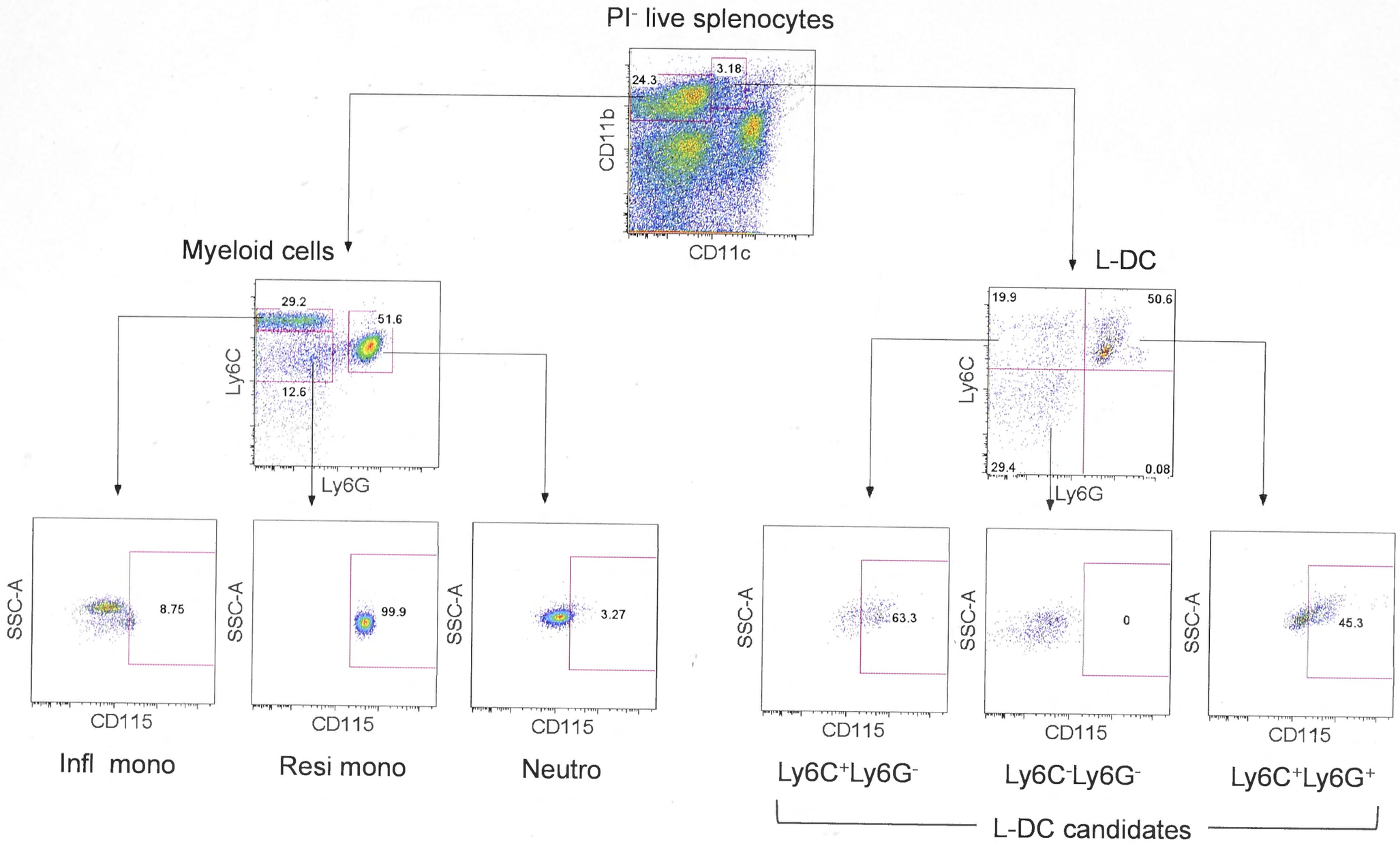
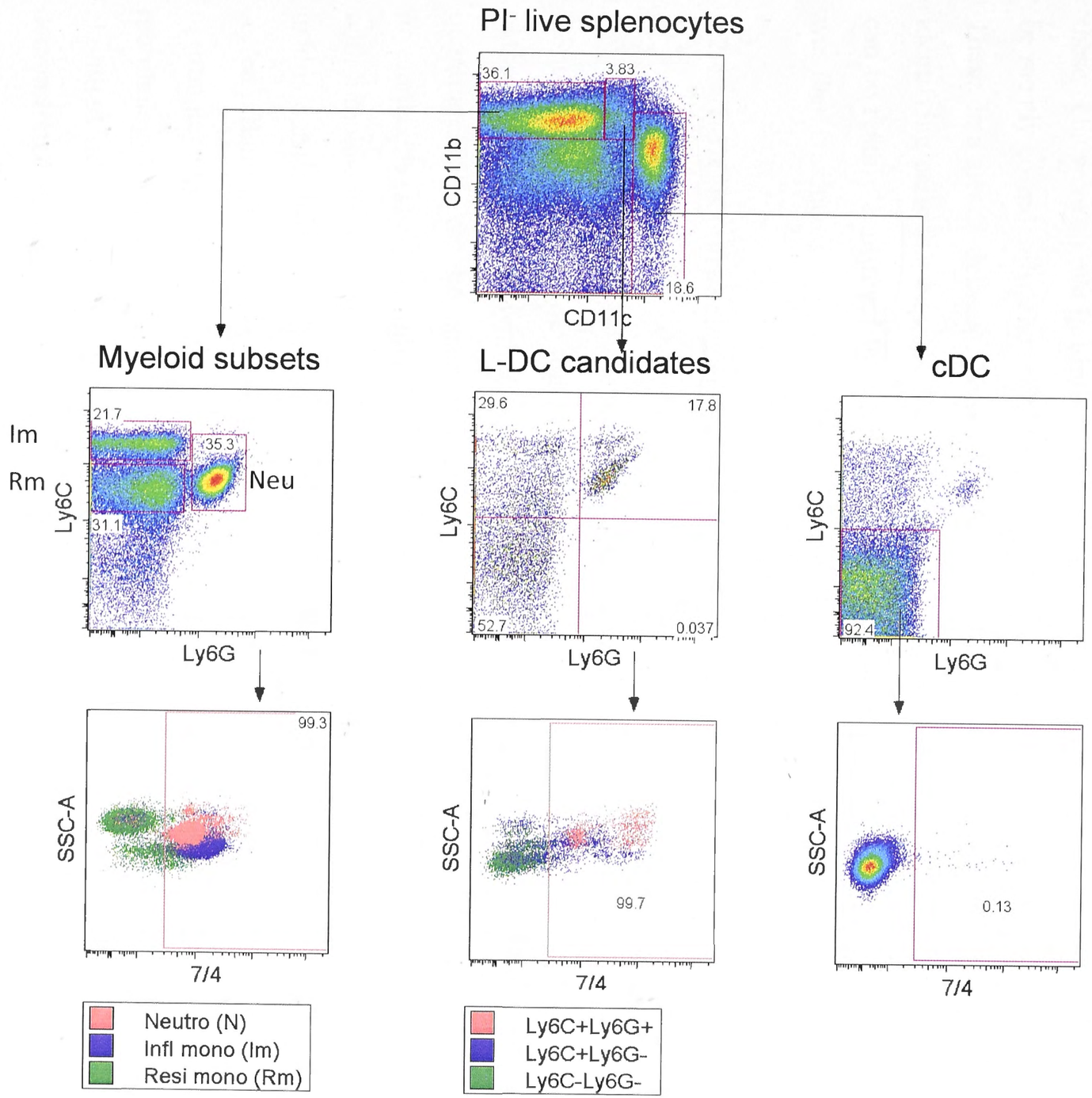

PI- live splenocytes
CD11b
CD11c
24.3
3.18
Myeloid cells
L-DC
Ly6C
Ly6G
29.2
51.6
12.6
19.9
50.6
29.4
0.08
SSC-A
CD115
8.75
99.9
3.27
63.3
0
45.3
Infl mono
Resi mono
Neutro
Ly6C+Ly6G-
Ly6C-Ly6G-
Ly6C+Ly6G+
L-DC candidates

**Figure 4.6 Further identification of splenic neutrophils.**

Splenocytes were prepared by red blood cell lysis and T/B cell depletion, and stained with antibodies specific for CD11b (M1/70, PE-Cy7), CD11c (N418, APC), Ly6C (Al-21, FITC), Ly6G (1A8, PE) and 7/4 (7/4, Biotin). Streptavidin (SA)-APC-Cy7 was used as a second stage reagent. Prior to analysis, cells were stained with propidium iodide (PI, 1μg/ml) for gating live ($PI^-$) cells. Myeloid subsets and L-DC candidates were gated as described in Figure 4.3, Im: inflammatory monocytes, Rm: resident monocytes and Neu : neutrophils. While cDC were gated as $CD11b^+CD11c^{hi}Ly6C^-Ly6G^-$ cells. Expression of the 7/4 marker of neutrophils was determined on all subsets, and data are presented as overlaid dot plot displays. Gates were set based on fluorescence minus one controls, and numbers in gates represent % specific binding.


PI- live splenocytes
36.1
3.83
18.6
CD11b
CD11c
Myeloid subsets
L-DC candidates
cDC
Im
Rm
21.7
35.3
Neu
31.1
Ly6C
Ly6G
29.6
17.8
52.7
0.037
92.4
99.3
SSC-A
7/4
99.7
0.13
Neutro (N)
Infl mono (Im)
Resi mono (Rm)
Ly6C+Ly6G+
Ly6C+Ly6G-
Ly6C-Ly6G-

positive control, while cDC served as appropriate negative controls (Figure 4.6). Most inflammatory monocytes (98%), but only a few resident monocytes (9.55%) showed 7/4 staining. The $Ly6C^{+}Ly6G^{+}$ L-DC candidate subset was uniformly positive for 7/4, indicating that this subset is probably neutrophils. However 80% of the cells in the $Ly6C^{+}Ly6G^{-}$ L-DC candidate subset were also positive, and since these cells were shown in Figure 4.3 to express Siglec-F, it is possible that they may be mostly eosinophils. The $Ly6C^{-}Ly6G^{-}$ L-DC candidate subset was negative for 7/4. These data clearly delineate both the myeloid and L-DC candidate subsets further by identifying cells that express 7/4. Since the presence of neutrophils and eosinophils can be readily confirmed by morphology, histological analysis of all sorted subsets was then conducted.

### 4.2.4 L-DC is morphologically distinct from splenic myeloid subsets

Myeloid and DC subsets were stained with antibodies as described in Figure 4.3 and then sorted flow cytometrically. Subsequently, sorted cells were prepared as cytospins and stained with Giemsa. Photomicroscopy was used to identify and quantitate cells on the basis of morphology. Gated $CD11b^{hi}CD11c^{-}Ly6C^{+}Ly6G^{+}$ myeloid cells (also $7/4^{+}$Siglec-$F^{-}$) demonstrated characteristic neutrophil morphology with a multi-lobate nucleus and cytoplasm devoid of granules (Figure 4.7). All cells in this subset had this characteristic morphology (Table 4.1). Giemsa staining of sorted inflammatory monocytes revealed a bi-lobate nucleus, and a cytoplasm devoid of granules typical of monocytes (Figure 4.7). The majority of cells (>90%) had this morphology (Table 4.1). Gated $CD11b^{hi}CD11c^{-}Ly6C^{+}Ly6G^{-}$ cells, originally classified as resident monocytes, now appear to be clearly eosinophils. All cells demonstrated multi-lobate nuclei with the clear presence of orange granules in the cytoplasm. This morphology is very consistent with eosinophils, and 100% of this sorted subset had this similar form (Table 4.1). In contrast, the $Ly6C^{+}Ly6G^{-}$ L-DC candidate subset revealed morphology resembling monocytes with a bi-lobate nucleus and a cytoplasm devoid of granules (Figure 4.7). However, this subset was also heterogeneous, comprising only ~65% mononuclear cells or monocytes (Table 4.1). The $Ly6C^{+}Ly6G^{+}$ L-DC candidate subset was found to be a mixture comprising mainly neutrophils with ~10% monocytes (data not shown). Both $CD8^{+}$ cDC and $CD8^{-}$ cDC displayed similar morphology and characteristics as DC. A majority (80-

**Figure 4.7 Morphology of splenic dendritic and myeloid subsets.**

Splenocytes were prepared by red blood cell lysis and T/B cell depletion, and stained with antibodies specific for CD11b (M1/70, Pe-Cy7), CD11c (N418, APC), Ly6C (AL-21, Pacific Blue), Ly6G (1A8, Bio), CD43 (IBII, Alexa488) and Siglec-F (E50-2440, PE), using streptavidin (SA)-APC-Cy7 as a second stage reagent. Prior to analysis, cells were stained with propidium iodide (PI, 1μg/ml) for gating live ($PI^-$) cells. Myeloid subsets, cDC and L-DC candidates were gated as described in Figure 4.3 for sorting. CD43 and Siglec-F staining were also used to delineate subsets, but gating on these antibodies is not shown. Gates were set based on fluorescence minus one controls, and numbers in gates represent % specific binding. Sorted subsets were cytospun on to slides and fixed with absolute ethanol prior to Giemsa staining to elucidate morphology microscopically. Photomicroscopy of subsets was performed at 600X magnification, bar 10μm.

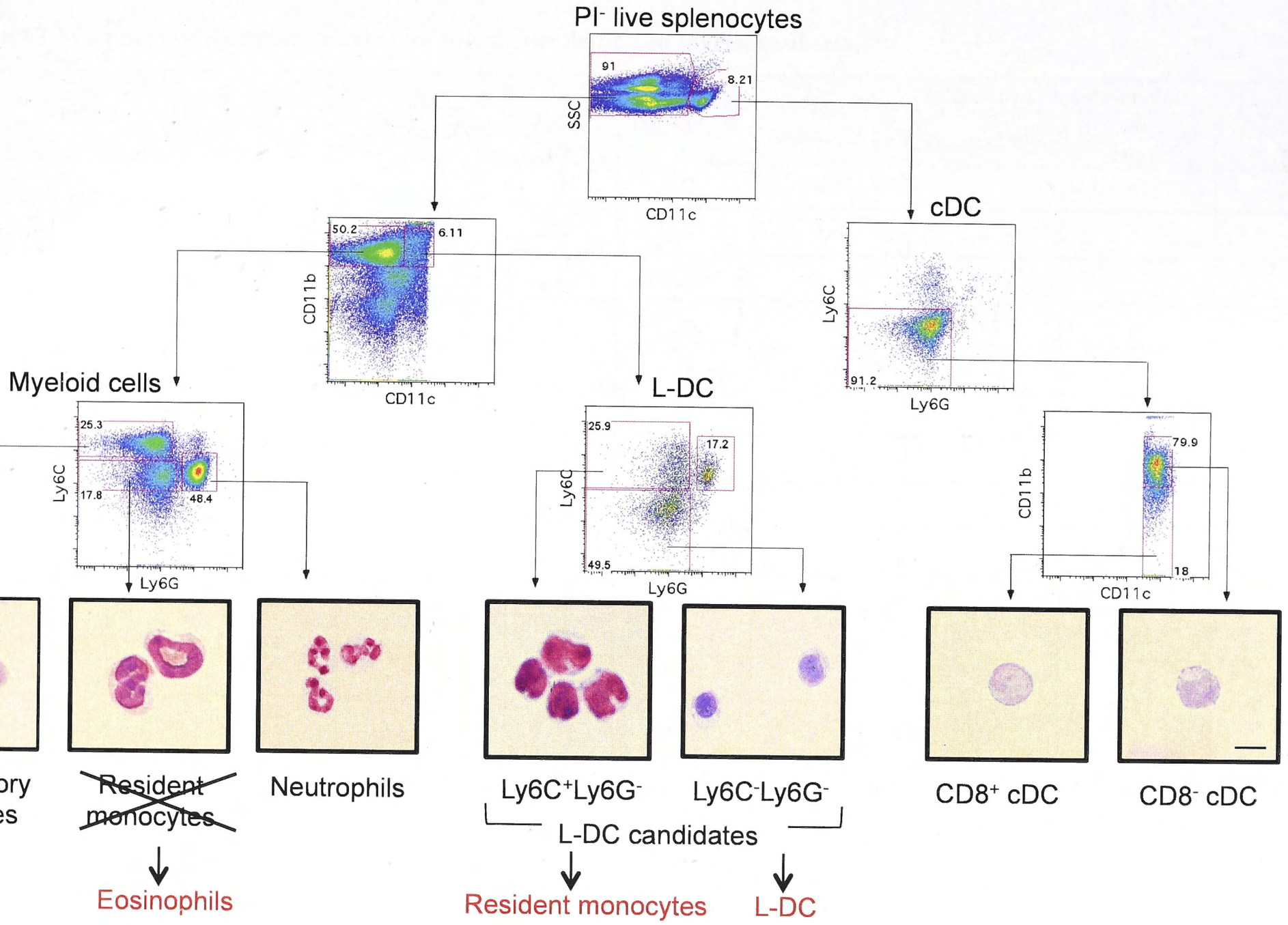

PI- live splenocytes
91
8.21
SSC
CD11c
50.2
6.11
CD11b
CD11c
Myeloid cells
25.3
17.8
48.4
Ly6C
Ly6G
Inflammatory monocytes
Resident monocytes
Neutrophils
Eosinophils
L-DC
25.9
17.2
49.5
Ly6C
Ly6G
Ly6C+Ly6G-
Ly6C-Ly6G-
L-DC candidates
Resident monocytes
L-DC
cDC
Ly6C
91.2
Ly6G
79.9
CD11b
18
CD11c
CD8+ cDC
CD8- cDC

**Table 4.1 Morphological characterisation of splenic dendritic and myeloid subsets.**

| Sorted subset | Subset classification | Expt* | No. of cells | % cells with known morphology+ | | | |
|---|---|---|---|---|---|---|---|
| | | | | Eosinophils | Neutrophils | Monocyte-like | Dendritic-like |
| $CD11b^{hi}CD11c^{-}Ly6C^{+}Ly6G^{-}CD43^{hi}Siglec\text{-}F^{+}$ | Eosinophils | I | 72 | **100**# | - | - | - |
| | | II | 72 | **100** | - | - | - |
| $CD11b^{hi}CD11c^{-}Ly6C^{+}Ly6G^{+}CD43^{+}Siglec\text{-}F^{-}$ | Neutrophils | I | 26 | - | **100** | - | - |
| | | II | 18 | - | **100** | - | - |
| $CD11b^{hi}CD11c^{-}Ly6C^{hi}Ly6G^{-}CD43^{+}Siglec\text{-}F^{-}$ | Inflammatory Monocytes | I | 72 | 1.4 | - | **97.2** | 1.4 |
| | | II | 68 | 1.5 | - | **86.8** | 11.8 |
| $CD11b^{hi}CD11c^{lo}Ly6C^{+}Ly6G^{-}CD43^{+/hi}Siglec\text{-}F^{-}$ | Resident Monocytes | I | 76 | - | - | **68.4** | 31.6 |
| | | II | 80 | - | - | **62.5** | 37.5 |
| $CD11b^{hi}CD11c^{lo}Ly6C^{-}Ly6G^{-}CD43^{+}Siglec\text{-}F^{-}$ | L-DC | I | 78 | 10.3 | - | 42.3 | **47.4** |
| | | II | 84 | 6.0 | - | 32.1 | **62** |
| $CD11b^{-}CD11c^{hi}Ly6C^{-}Ly6G^{-}CD43^{-}Siglec\text{-}F^{-}$ | $CD8^{+}$ cDC | I | 41 | - | - | 26.8 | **73.2** |
| | | II | 34 | - | - | 17.7 | **82.4** |
| $CD11b^{+}CD11c^{hi}Ly6C^{-}Ly6G^{-}CD43^{-}Siglec\text{-}F^{-}$ | $CD8^{-}$ cDC | I | 61 | - | - | 13.1 | **86.9** |
| | | II | 59 | 1.7 | - | 15.3 | **83.1** |

* Data are shown for 2 independent sorting experiments.
\+ Cells were cytospun and stained with Giemsa for microscopic classification.
\# Dominant subsets are shown in bold.

90%) of cells displayed the morphology of mononuclear cells with vacuoles evident in the cytoplasm, and nuclei staining bluish instead of red (Table 4.1, Figure 4.7).

In line with its proposed dendritic-like phenotype, the $Ly6C^{-}Ly6G^{-}$ L-DC candidate subset contained a majority of cells with the same morphology as the cDC subsets, with 47% and 62% of cells being mononuclear cells with vacuoles evident in the cytoplasm across two experiments (Table 4.1, Figure 4.7). This subset was therefore heterogeneous, containing 30-40% monocyte-like cells, and 5-10% eosinophils (Table 4.1). Overall, Giemsa staining served to distinguish the L-DC candidate subsets further, and to reveal the dendritic-like morphology of the majority of cells in the $Ly6C^{-}Ly6G^{-}$ L-DC candidate subset. It also disputed the monocytic nature of the classified resident monocyte subset, which appears to represent a clear eosinophil subset in spleen. Resident monocytes may instead represent the major subpopulation of cells present in the $Ly6C^{+}Ly6G^{-}$ L-DC candidate subset.

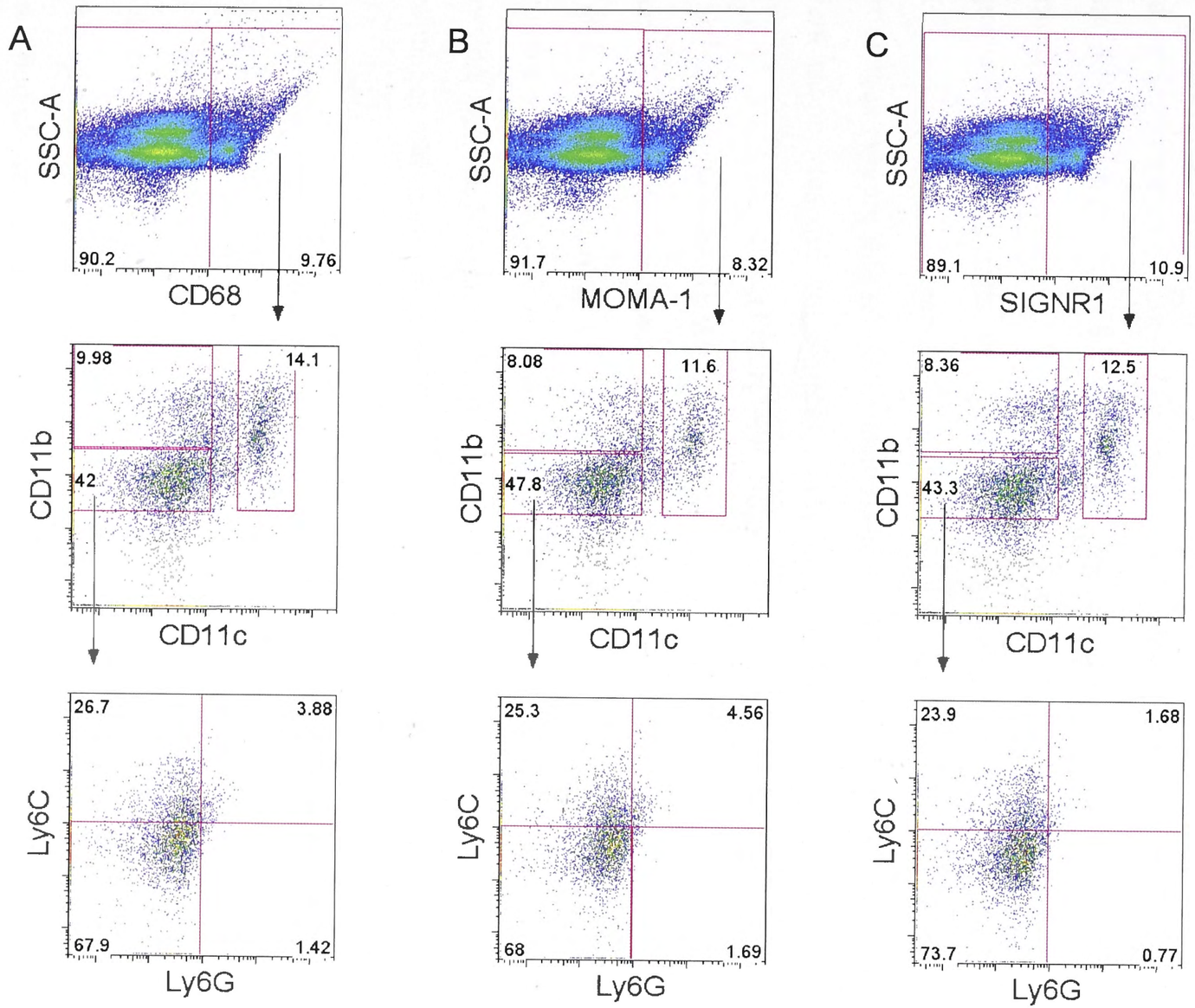
### 4.2.5 L-DC are distinct from splenic macrophages

Macrophages have been historically characterised in spleen based on immunohistological analysis. However, while some macrophage-specific markers have been identified, macrophage phenotypes have not been well characterised. In terms of testing whether the L-DC candidate subsets were contaminated with macrophages, it was first essential to determine the full phenotype of known splenic macrophages and to compare this with L-DC and other described subsets. Expression of the macrophage markers MOMA-1, SIGNR1 and CD68 was therefore investigated on splenocytes. MOMA-1 is specifically expressed by marginal zone metallophilic macrophages (MMM), SIGNR1 is expressed by marginal zone macrophages (MZM), and CD68 is expressed by white pulp macrophages (WPM) (Elomaa et al., 1995; Kang et al., 2003; Mebius and Kraal, 2005; Noel et al., 2007).

Spleen cells were stained for specific macrophage markers as well as CD11c, CD11b, Ly6C and Ly6G, and then gated as described in Figure 4.7. For each of the three macrophage markers, marker positive cells were gated and found to display a majority phenotype (40-50%) as $CD11b^{+}CD11c^{-}Ly6C^{+/-}Ly6G^{-}$ cells (Figure 4.8). This subset was termed 'putative macrophages', and served as a positive control for

**Figure 4.8 Identification of splenic macrophages.**

Splenocytes were prepared by red blood cell lysis and T/B cell depletion and stained with antibodies specific for CD11b (M1/70, Pe-Cy7), CD11c (N418, APC), Ly6C (AL-21, FITC), Ly6G (1A8, PE), and specific macrophage markers: A) CD68, B) MOMA-1, and C) SIGNR1. Streptavidin (SA)-APC-Cy7 was used as a second stage reagent. Cells positive for specific macrophage markers were gated and further examined on the basis of CD11b and CD11c expression. The majority of gated cells were $CD11b^{+}CD11c^{-}$ myeloid cells. Ly6C and Ly6G expression were then examined on $CD11b^{+}CD11c^{-}$ myeloid cells. Gates were set based on fluorescence minus one controls, and numbers in gates represent % specific binding.


A
SSC-A
90.2
9.76
CD68
9.98
14.1
42
CD11b
CD11c
26.7
3.88
67.9
1.42
Ly6C
Ly6G
B
SSC-A
91.7
8.32
MOMA-1
8.08
11.6
47.8
CD11b
CD11c
25.3
4.56
68
1.69
Ly6C
Ly6G
C
SSC-A
89.1
10.9
SIGNR1
8.36
12.5
43.3
CD11b
CD11c
23.9
1.68
73.7
0.77
Ly6C
Ly6G

comparison with other subsets. However, amongst the macrophage marker positive cells, subsets of $CD11c^{hi}$ cells which reflect cDC were found representing ~1% of the total spleen cell population (Figure 4.8). Some $CD11b^{hi}CD11c^{lo}$ cells were also present which therefore justified more extensive analysis.

In subsequence experiments, myeloid subsets and L-DC candidates were gated as described in Figure 4.7 and tested for expression of macrophage markers. Figure 4.9 shows that CD68 did not stain the gated $CD11b^{hi}CD11c^{-}Ly6C^{hi}Ly6G^{-}$ inflammatory monocytes, the $CD11b^{hi}CD11c^{-}Ly6C^{lo}Ly6G^{-}$ subset of cells categorised as eosinophils in Figure 4.7, nor did it stain the $CD11b^{hi}CD11c^{-}Ly6C^{+}Ly6G^{+}$ subset of neutrophils. However, CD68 did stain about 20% of putative macrophages (Figure 4.9 A). Cells within the $CD11c^{lo}$ gate reflective of L-DC and resident monocytes did not express CD68, nor did the gated $CD11c^{hi}$ subsets of $CD8^{+}$ cDC and $CD8^{-}$ cDC (Figure 4.9). Some $CD68^{+}$ cells were evident amongst the $CD11c^{lo}$ subset, which expressed both Ly6C and Ly6G and resembled neutrophil-like cells. However, similar staining and analysis of spleen cells using MOMA-1 and SIGNR1 revealed staining to be specific for only a subset (~20%) of putative macrophages (Figure 4.10 A and 4.11 A). No other cells, including L-DC, showed expression of MOMA-1 or SIGNR1 specific for marginal zone metallophilic macrophages and marginal zone macrophages (Figure 4.10 B and 4.11 B).

Splenic macrophages can be divided into two populations based on their anatomical location, namely white pulp and red pulp macrophages. While antibodies against MOMA-1, SIGNR1 and CD68 can be used to detect subsets of marginal zone and white pulp macrophages, expression of F4/80 can be used to detect the presence of red pulp macrophages. Myeloid subsets and L-DC candidates were therefore gated as described above, and F4/80 staining was determined. Putative macrophages were gated as in Figure 4.8, and served as a positive control. A subset (27%) of the putative macrophages was positive for F4/80 (Figure 4.12A). Almost all of the inflammatory and resident monocytes were positive for F4/80, while only 21% of neutrophils expressed F4/80 (Figure 4.12B). Similarly, almost all of the $Ly6C^{+}Ly6G^{-}$ resident monocytes and $Ly6C^{-}Ly6G^{-}$ L-DC subsets stained for F4/80, while 74% of the $Ly6C^{+}Ly6G^{+}$ L-DC candidate, reflective of neutrophil-like cells, stained for F4/80. To determine the specificity of F4/80 staining, both subsets of cDC were

**Figure 4.9 L-DC is distinct from splenic white pulp macrophages.**

Splenocytes were prepared by red blood cell lysis and T/B cell depletion, and stained with antibodies specific for CD11b (M1/70, PE-Cy7), CD11c (N418, APC), Ly6C (AL-21, FITC), Ly6G (1A8, PE) and F4/80 (CI:A3-1, Biotin). Streptavidin (SA)-APC-Cy7 was used as a second stage reagent. L-DC candidates, and myeloid and DC subsets, were gated as described in Figure 4.3, while putative macrophages were gated as $CD11b^{+}CD11c^{-}Ly6C^{-}Ly6G^{-}$ cells. Specific antibody to CD68 was used to identify white pulp macrophages. A) Expression of CD68 on myeloid subsets and putative macrophages. B) Expression of CD68 on L-DC candidates and cDC subsets. Gates were set based on fluorescence minus one controls, and numbers in gates represent % specific binding.

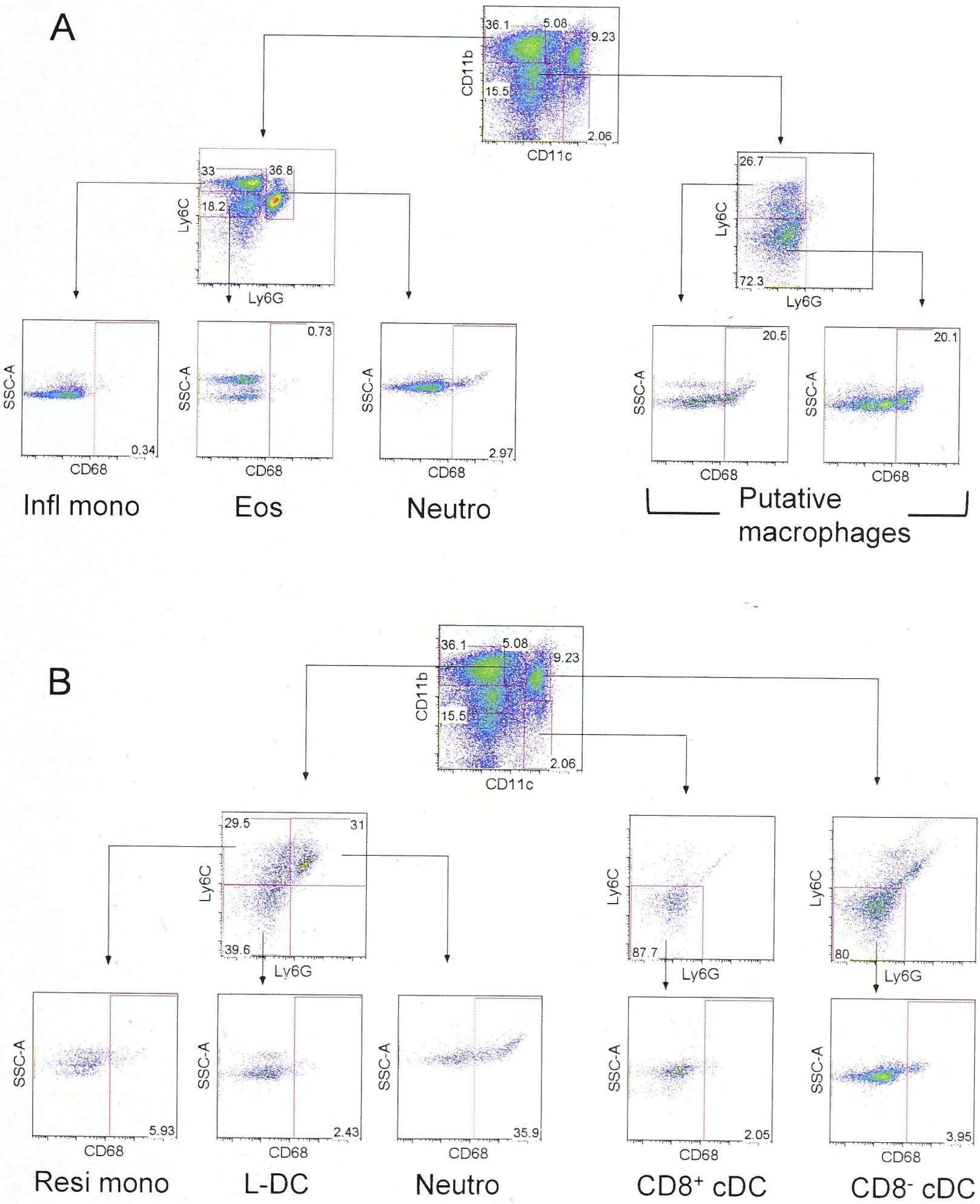
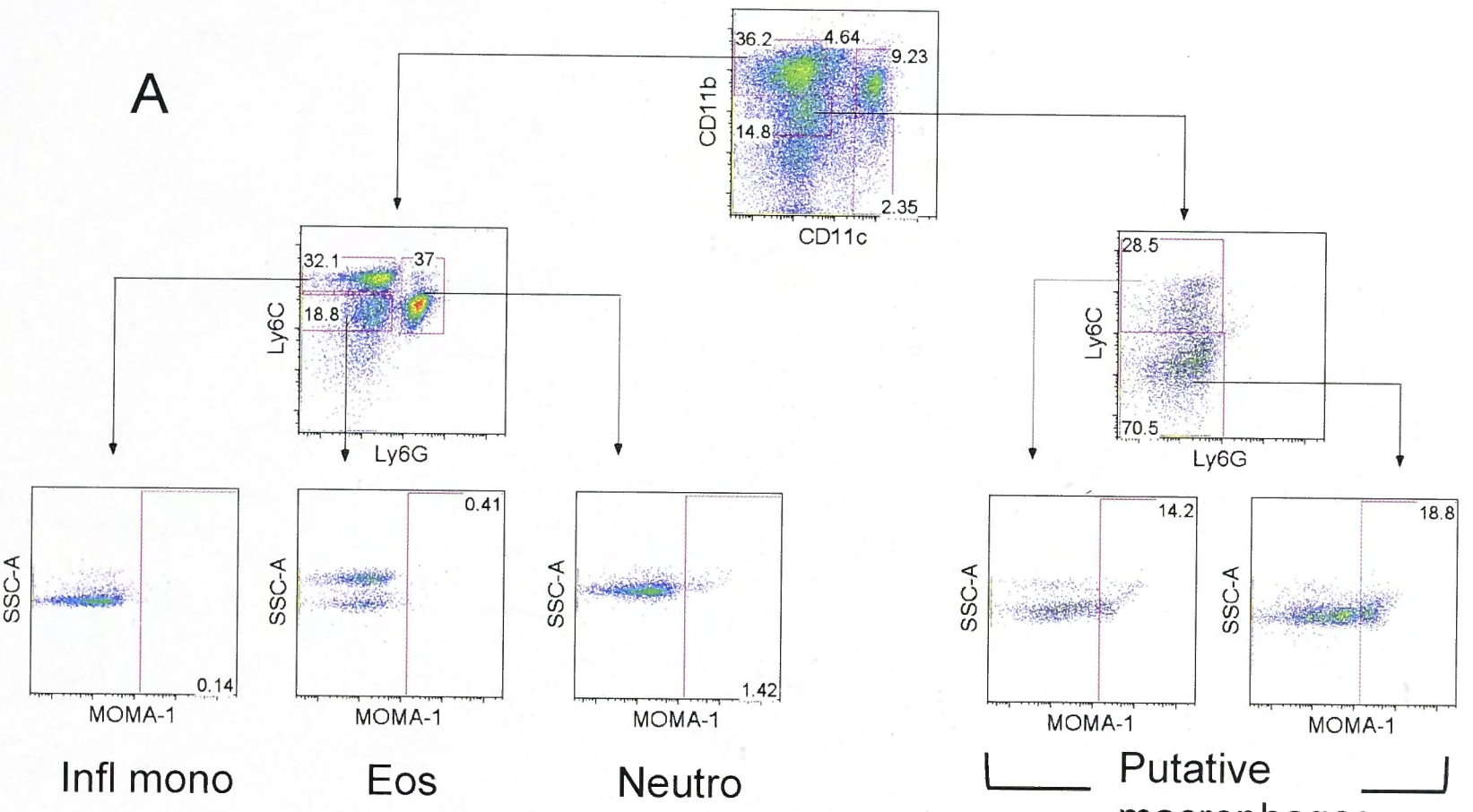

A
CD11b
CD11c
36.1
5.08
9.23
15.5
2.06
Ly6C
Ly6G
33
36.8
18.2
26.7
72.3
SSC-A
CD68
0.34
0.73
2.97
20.5
20.1
Infl mono
Eos
Neutro
Putative
macrophages
B
29.5
31
39.6
87.7
80
5.93
2.43
35.9
2.05
3.95
Resi mono
L-DC
Neutro
CD8+ cDC
CD8- cDC

**Figure 4.10 L-DC is distinct from splenic marginal-zone metallophilic macrophages.**

Splenocytes were prepared by red blood cell lysis and T/B cell depletion, and stained with antibodies specific for CD11b (M1/70, PE-Cy7), CD11c (N418, APC), Ly6C (AL-21, FITC), Ly6G (1A8, PE) and F4/80 (CI:A3-1, Biotin). Streptavidin (SA)-APC-Cy7 was used as a second stage reagent. L-DC candidates, and myeloid and DC subsets, were gated as described in legend to Figure 4.3, while putative macrophages were gated as $CD11b^{+}CD11c^{-}Ly6C^{-}Ly6G^{-}$ cells. Specific antibody to MOMA-1 was used to identify marginal-zone metallophilic macrophages. A) Expression of MOMA-1 on myeloid subsets and putative macrophages. B) Expression of MOMA-1 on L-DC candidates and cDC subsets. Gates were set based on fluorescence minus one controls, and numbers in gates represent % specific binding.


A
CD11b
CD11c
36.2
4.64
9.23
14.8
2.35
Ly6C
Ly6G
32.1
37
18.8
28.5
70.5
SSC-A
MOMA-1
0.14
0.41
1.42
14.2
18.8
Infl mono
Eos
Neutro
Putative
macrophages

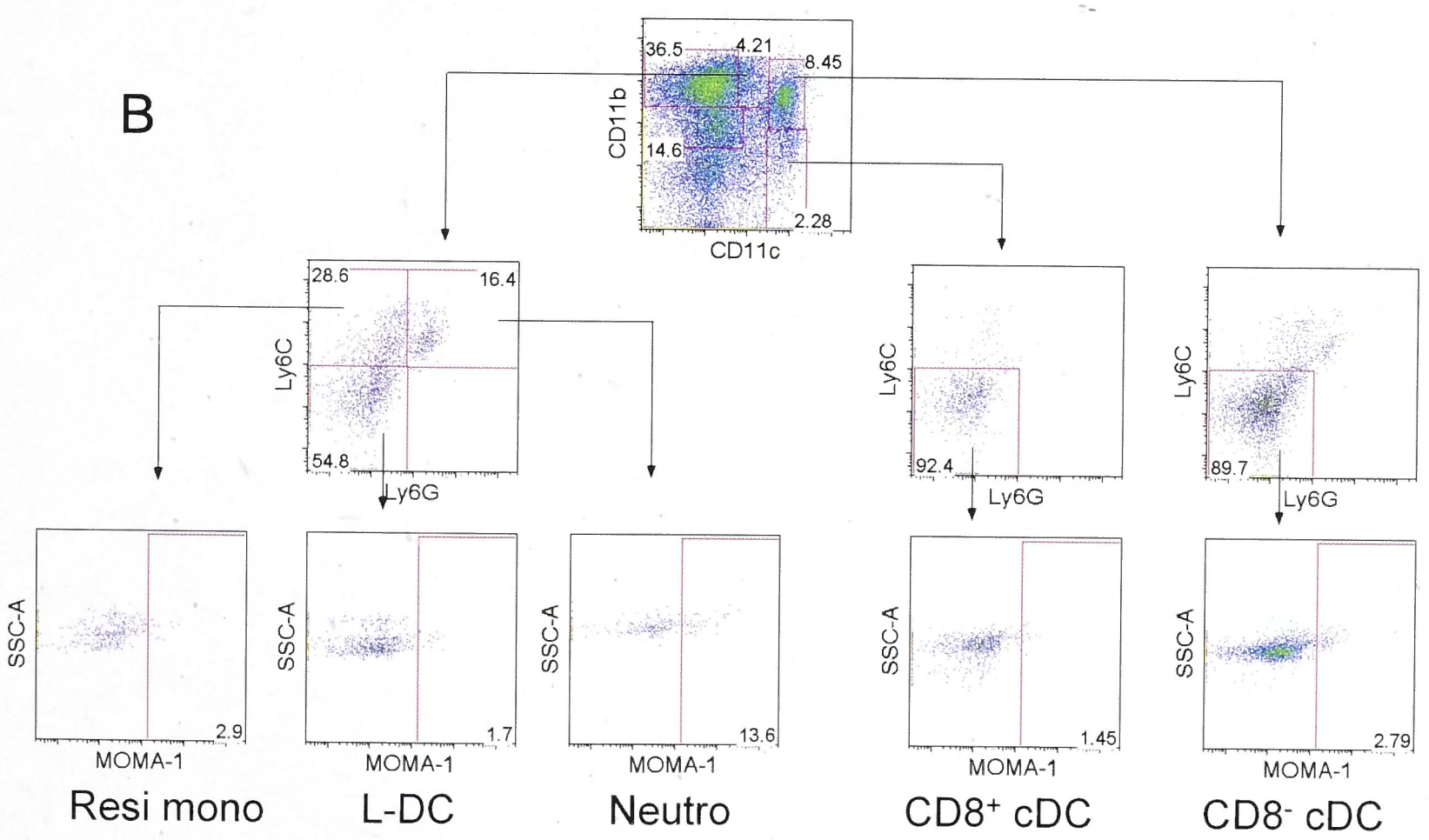
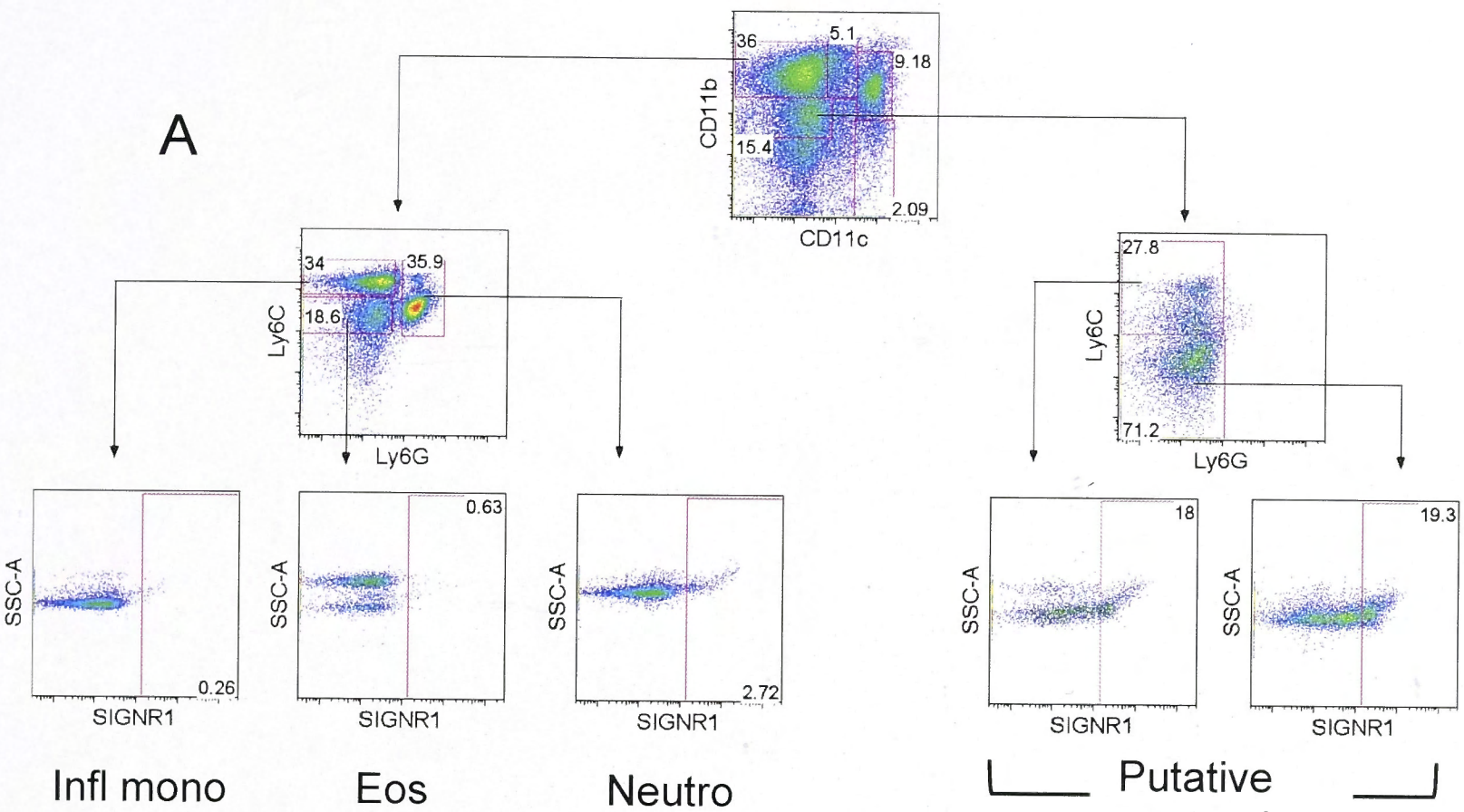

B
CD11b
CD11c
36.5
4.21
8.45
14.6
2.28
Ly6C
Ly6G
28.6
16.4
54.8
92.4
89.7
SSC-A
MOMA-1
2.9
1.7
13.6
1.45
2.79
Resi mono
L-DC
Neutro
CD8+ cDC
CD8- cDC

**Figure 4.11 L-DC is distinct from spleen marginal zone macrophages.**

Splenocytes were prepared by red blood cell lysis and T/B cell depletion, and stained with antibodies specific for CD11b (M1/70, PE-Cy7), CD11c (N418, APC), Ly6C (AL-21, FITC), Ly6G (1A8, PE) and F4/80 (CI:A3-1, Biotin). Streptavidin (SA)-APC-Cy7 was used as a second stage reagent. L-DC candidates, and myeloid cells and DC subsets were gated as described in the legend to Figure 4.3, while putative macrophages were gated as $CD11b^{+}CD11c^{-}Ly6C^{-}Ly6G^{-}$ cells. Specific antibody to SIGNR1 was used to identify marginal zone macrophages. A) Expression of SIGNR1 on myeloid subsets and putative macrophages. B) Expression of SIGNR1 on L-DC candidates and cDC subsets. Gates were set based on fluorescence minus one controls, and numbers in gates represent % specific binding.


A
CD11b
CD11c
36
5.1
9.18
15.4
2.09
Ly6C
Ly6G
34
35.9
18.6
27.8
71.2
SSC-A
SIGNR1
0.26
0.63
2.72
18
19.3
Infl mono
Eos
Neutro
Putative
macrophages

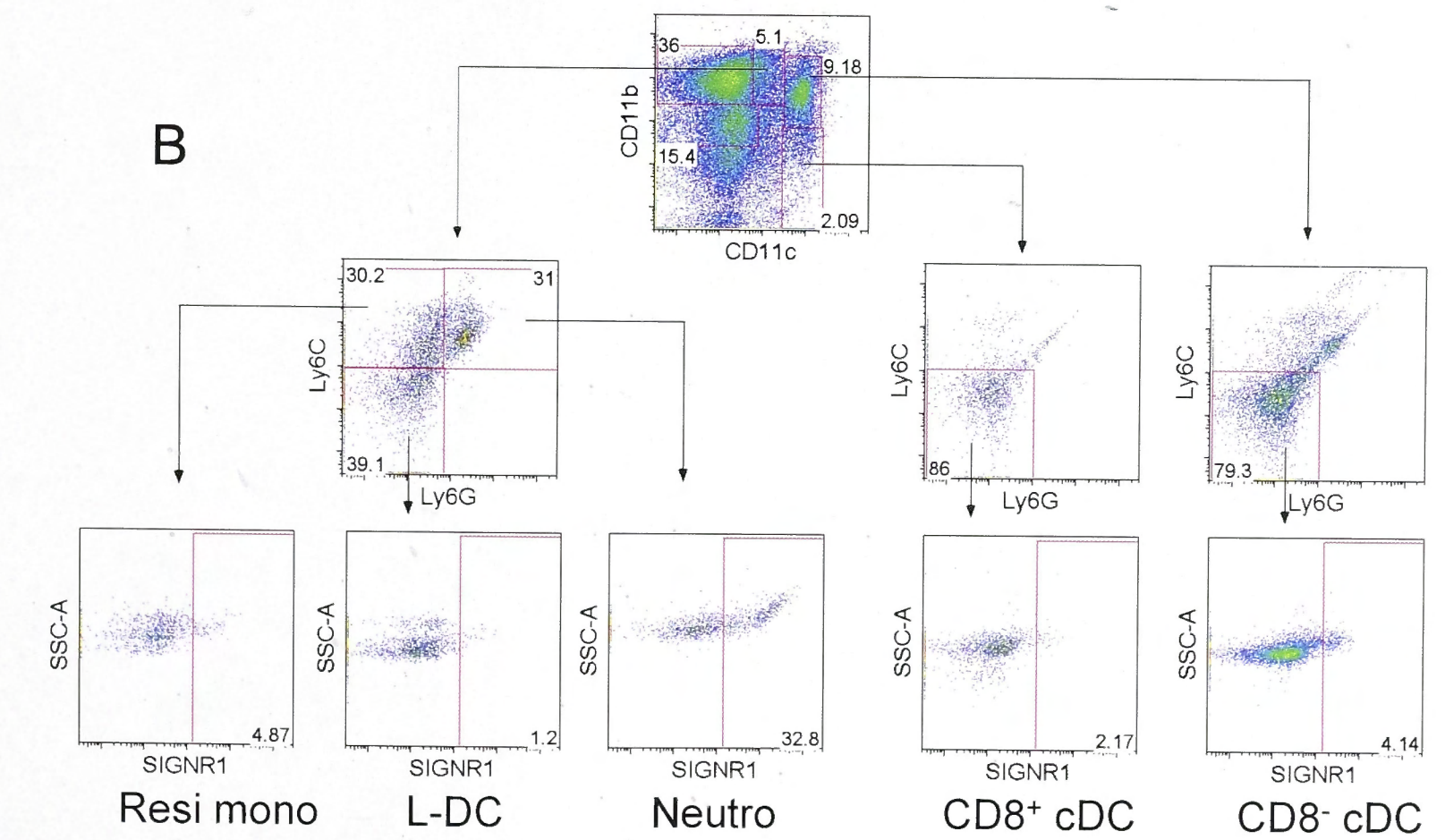
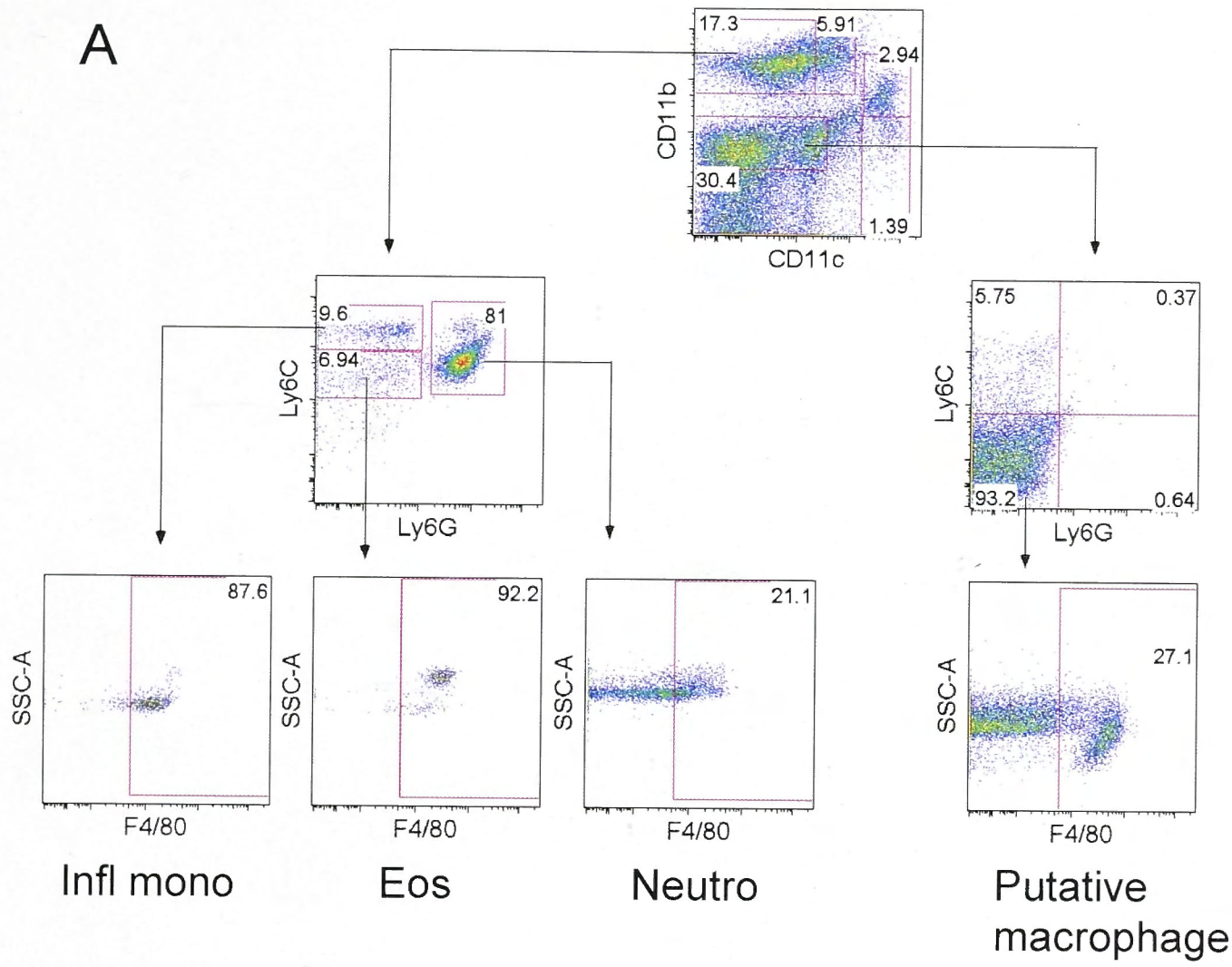

B
CD11b
CD11c
36
5.1
9.18
15.4
2.09
Ly6C
Ly6G
30.2
31
39.1
86
79.3
SSC-A
SIGNR1
4.87
1.2
32.8
2.17
4.14
Resi mono
L-DC
Neutro
CD8+ cDC
CD8- cDC

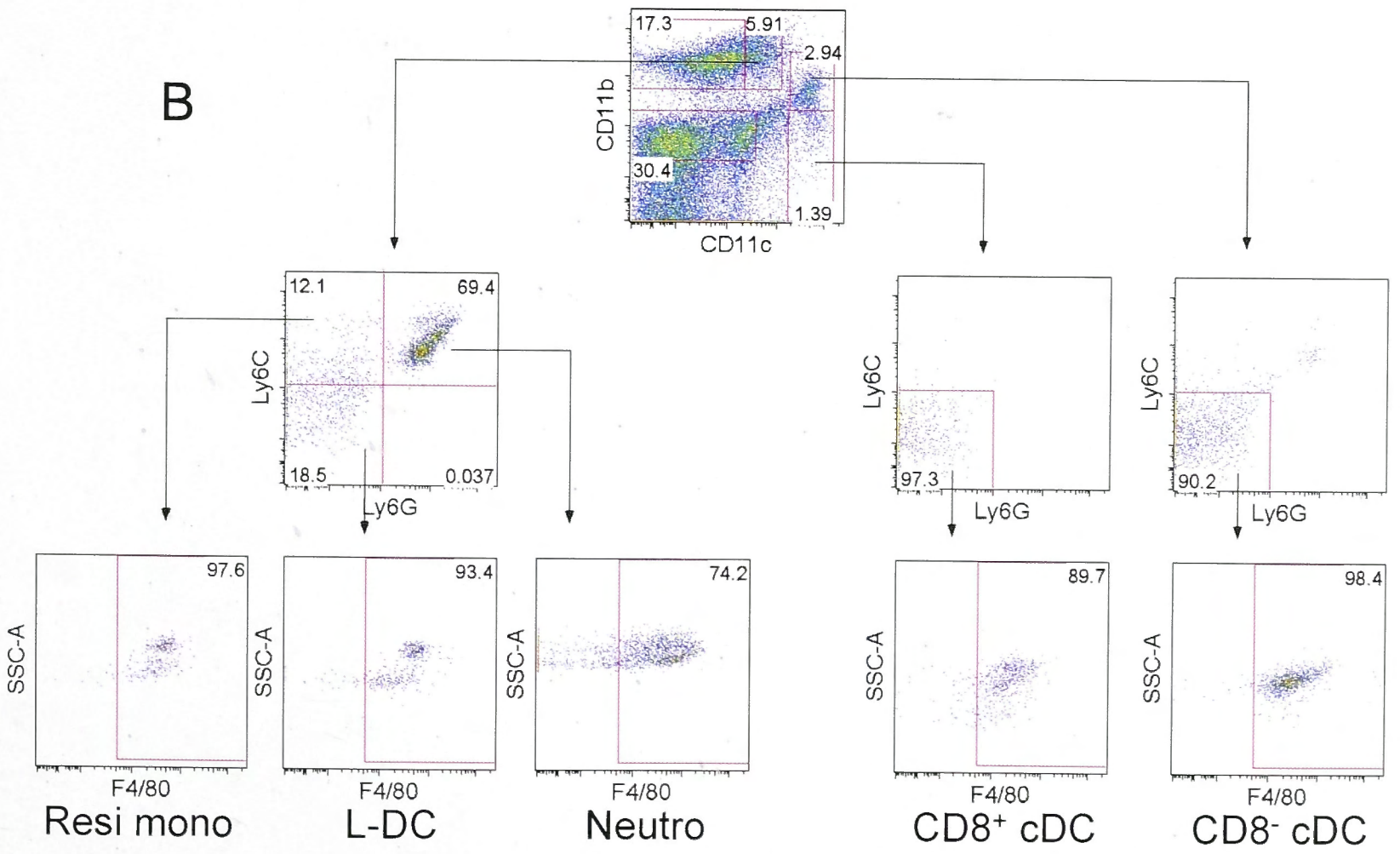
**Figure 4.12 Identification of splenic red pulp macrophages.**

Splenocytes were prepared by red blood cell lysis and T/B cell depletion and stained with antibodies specific for CD11b (M1/70, PE-Cy7), CD11c (N418, APC), Ly6C (AL-21, FITC), Ly6G (1A8, PE) and F4/80 (CI:A3-1, Biotin). Streptavidin (SA)-APC-Cy7 was used as a second stage reagent. L-DC candidates, myeloid cells and DC subsets were gated as described in the legend to Figure 4.3, while putative macrophages were gated as $CD11b^{+}CD11c^{-}Ly6C^{-}Ly6G^{-}$ cells. A) Expression of F4/80 on myeloid subsets and putative macrophages. B) Expression of F4/80 on L-DC candidates and cDC subsets. Gates were set based on fluorescence minus one controls, and numbers in gates represent % specific binding.

A


17.3
5.91
2.94
30.4
1.39
CD11b
CD11c
9.6
81
6.94
Ly6C
Ly6G
5.75
0.37
93.2
0.64
87.6
92.2
21.1
27.1
SSC-A
F4/80
Infl mono
Eos
Neutro
Putative macrophages

B


17.3
5.91
2.94
30.4
1.39
CD11b
CD11c
12.1
69.4
18.5
0.037
Ly6C
Ly6G
97.3
90.2
97.6
93.4
74.2
89.7
98.4
SSC-A
F4/80
Resi mono
L-DC
Neutro
CD8+ cDC
CD8- cDC

gated and expression of F4/80 determined. Almost 100% of gated cDC were F4/80$^{+}$, indicating that F4/80 can be expressed by both myeloid and DC subsets in spleen.

## 4.3 Discussion

L-DC have been described as myeloid DC in that they express high levels of CD11b. It was therefore important to explore the relationship between L-DC and other known CD11b-expressing myeloid subsets in spleen. In order to compare L-DC with myeloid subsets, it was first essential to clearly identify all known myeloid subsets within spleen. Thus, a broad screen of cell surface marker expression and morphological analysis was performed to develop a staining and gating strategy for accurate identification of all myeloid and DC subsets. This was based on both existing information in the literature, and our own laboratory experience in splenic subset analysis.

This analysis first distinguished the $CD8^+$ cDC and $CD8^-$ cDC subsets, which are already well defined in the literature. Identification of inflammatory monocytes and neutrophils was also straightforward according to published information. Resident monocytes were more difficult to define and required broader comparative analysis. The L-DC subset was first identified in terms of three candidates, two of which were found to resemble resident monocytes and neutrophils, allowing delineation of the most likely L-DC candidate.

From the experimental data shown here, cells in spleen with the phenotype of resident monocytes described in the literature, showed more resemblance to eosinophils than to monocytes. This conclusion was based on both cell surface marker expression of Siglec-F as well as cell morphology studies. Phenotypic analysis of $CX_3CR1$ and CD43 expression, along with morphological analysis, also revealed that the $Ly6C^+Ly6G^-$ L-DC candidate subset showed properties of resident monocytes similar to that described in the literature. We also showed on the basis of 7/4 expression that the $Ly6C^+Ly6G^+$ L-DC candidate reflected neutrophils. Lastly, on the basis of phenotypic and morphological studies, the $Ly6C^-Ly6G^-$ L-DC candidate was identified as the best match for an *in vivo* equivalent of LTC-DC.

The current classification and naming of myeloid subsets is based on accumulated flow cytometric data and various functional assays. Blood monocytes are the most widely studied and characterised monocytes, although splenic

monocytes have been shown to closely resemble their blood counterparts in terms of phenotype, function and gene expression (Jung et al., 2000; Voehringer et al., 2007). Inflammatory blood monocytes were previously described as the $Ly6C^{hi}$ subset of $CD11b^{hi}CD11c^{-}MHCII^{-}Ly6G^{-}$ cells, with resident blood monocytes as the $Ly6C^{lo}$ equivalent subset (Auffray et al., 2007; Geissmann et al., 2003; Gordon and Taylor, 2005; Sunderkötter et al., 2004; Tacke et al., 2007). In line with the literature, we attempted to define both splenic inflammatory and resident monocytes in spleen based on the phenotype of their blood counterpart (Figure 4.1). However, multiple analyses revealed that the putative resident monocyte subset in spleen having a $CD11b^{hi}CD11c^{-}MHCII^{-}Ly6C^{lo}Ly6G^{-}$ phenotype, differed from its blood resident monocyte counterpart. The splenic subset equivalent to resident monocytes in blood was instead enriched for eosinophils. Splenic neutrophils were easily identifiable as $CD11b^{hi}CD11c^{-}MHCII^{-}Ly6C^{+}Ly6G^{+}$ cells, as described in the literature (Fleming et al., 1993; Sumagin et al., 2010). We also showed that it is possible to further distinguish splenic monocyte subsets from neutrophils by their distinct side scatter (SSC) and forward scatter (FSC) profiles (Figure 4.1).

L-DC were thought to resemble myeloid dendritic-like cells based on their $CD11b^{hi}CD11c^{lo}MHCII^{-}$ phenotype. To distinguish these cells from monocytes, and to avoid subset contamination, Ly6C and Ly6G expression were first employed. L-DC were gated as $CD11b^{hi}CD11c^{lo}MHCII^{-}$ cells, and then further delineated on the basis of Ly6C and Ly6G expression (Figure 4.2). Three L-DC candidate populations were identified initially, namely $Ly6C^{+}Ly6G^{+}$, $Ly6C^{+}Ly6G^{-}$ and $Ly6C^{-}Ly6G^{-}$ cells (Figure 4.2). SSC and FSC profiles also served to distinguish these subsets. The $Ly6C^{+}Ly6G^{+}$ L-DC candidate displayed a $SSC^{mid}FSC^{hi}$ profile, resembling neutrophils (Figures 4.1 and 4.2), while the $Ly6C^{-}Ly6G^{-}$ L-DC candidate displayed a $SSC^{lo}FSC^{lo}$ profile, distinct from $SSC^{hi}$ inflammatory monocytes (Figures 4.1 and 4.2). Lastly, the SSC/FSC profile of the $Ly6C^{+}Ly6G^{-}$ L-DC candidate revealed a heterogeneous population that overlapped with the $Ly6C^{+}Ly6G^{+}$ and $Ly6C^{-}Ly6G^{-}$ L-DC candidate subsets (Figure 4.2). SSC profiles suggested some myeloid cell contamination within the L-DC candidate subsets. For this reason, more myeloid markers were employed to delineate L-DC further. Those myeloid markers also served to better define other splenic myeloid subsets.

Both inflammatory and resident monocytes in blood are thought to derive from a CMP in bone marrow (Fogg et al., 2006; van Furth et al., 1972), and hence display similar markers and requirements for development. The expression of the CD43, $CX_3CR_1$ and Ly6C markers were therefore used to distinguish resident and inflammatory monocytes. Previously, resident monocytes were characterised as $Ly6C^{+}CD43^{hi}CX_3CR1^{hi}$ cells, while inflammatory monocytes are distinct as $Ly6C^{hi}CD43^{+}CX_3CR1^{lo}$ cells (Auffray et al., 2007; Geissmann et al., 2003; Sunderkötter et al., 2004). In studies described here, all splenic myeloid subsets were found to express CD43, although resident monocytes were evident as a distinct $CD43^{hi}$ sub-population (Figure 4.3). The $Ly6C^{+}Ly6G^{-}$ L-DC candidate subset was also found to contain ~37% $CD43^{hi}$ cells, consistent with the presence of resident monocytes (Figure 4.3). Others have however distinguished resident monocytes as $CD43^{+}$ cells, and inflammatory monocytes as $CD43^{-}$ cells (Geissmann et al., 2003; Sunderkötter et al., 2004). Inconsistency between the literature and the results with CD43 staining described here could be attributable to the nature of the controls used to measure CD43 expression. Sunderkötter et al. (2004) used isotype controls, while fluorescence minus one controls were employed here to delineate CD43 expression. In addition, Sunderkötter et al. (2004) only compared CD43 expression amongst blood monocytes. In staining experiments shown here, cDC as $CD43^{-}$ cells, were used as an internal biological control for CD43 staining (Figure 4.3 A).

To directly test whether the $Ly6C^{+}Ly6G^{-}$ L-DC candidate subset was contaminated with resident monocytes, several markers of monocytes including $CX_3CR1$ were assessed for expression on myeloid and DC subsets. Mice transgenic for $CX_3CR1$ expression tagged with green fluorescence protein (GFP), allowed detection of cells that descend from the macrophage/dendritic lineage (Fogg et al., 2006; Nahrendorf et al., 2007; Swirski et al., 2009). Auffray et al. (2007) defined resident monocytes as $CX_3CR1^{hi}$ cells and inflammatory monocytes as $CX3CR1^{lo}$ cells. Contrary to the literature, staining experiments here revealed that the $Ly6C^{+}Ly6G^{-}SSC^{hi}$ subset of 'apparent' resident monocytes in spleen lacked $CX_3CR1$ expression (Figure 4.4), while $Ly6C^{hi}Ly6G^{-}SSC^{lo}$ inflammatory monocytes were $CX_3CR1^{lo}$ (Figure 4.4), consistent with the literature. As with inflammatory monocytes, the $Ly6C^{-}Ly6G^{-}$ L-DC candidate was also $CX_3CR1^{lo}$. Since the $Ly6C^{+}Ly6G^{-}$ L-DC candidate was found to contain a distinct $CX_3CR1^{hi}$ subset of

cells (63%, Figure 4.4), these cells appear to more closely resemble resident monocytes. They are, however, a subset showing low expression of CD11c, consistent with studies reporting that monocytes and some macrophages do express low levels of CD11c (Drutman et al., 2012; Gower et al., 2011; Soos et al., 2006; Vallon-Eberhard et al., 2006). Analysis of resident monocytes by Auffray et al. (2007) examined only CD11b, Ly6C and Ly6G expression, and not CD11c expression on monocytes. The $CX_3CR1^{hi}$ subset of resident monocytes therefore resembles the $Ly6C^+Ly6G^-$ L-DC candidate. If this subset is indeed 'resident monocytes', then the identity of the literature described $CD11b^{hi}CD11c^-MHCII^-Ly6C^+Ly6G^-$ subset of resident monocytes in spleen needs to be revisited.

To further examine the identity of the $CD11b^{hi}CD11c^-MHCII^-Ly6C^+Ly6G^-$ subset, several new cell surface markers were analysed. Siglec-F, an inhibitory receptor expressed by murine eosinophils (Bochner, 2009; Guo et al., 2007) proved definitive for subset identification (Figure 4.3 B). The $CD11b^{hi}CD11c^-MHCII^-Ly6C^+Ly6G^-$ subset of reputed resident monocytes contained a major subpopulation of Siglec-$F^+$ cells reflecting eosinophils (87%, Figure 4.3), while inflammatory monocytes were Siglec-$F^-$ cells (~99%, Figure 4.3). The $Ly6C^+Ly6G^-$ and $Ly6C^-Ly6G^-$ L-DC candidate subsets were mainly Siglec-$F^-$ cells. Overall, Siglec-$F^+$ eosinophils were mapped to the $CD11b^{hi}CD11c^-MHCII^-Ly6C^+Ly6G^-$ population. From the above results, resident monocytes formally described in the literature as $CD11c^-$ cells reflect a population heavily contaminated with eosinophils, and resident monocytes in spleen appear to lie within the $Ly6C^+Ly6G^-$ candidate L-DC population expressing $CD11c^{lo}$.

To further test these findings, more myeloid markers like CD115 were employed (Dai et al., 2002; Sasmono et al., 2003; Wiktor-Jedrzejczak and Gordon, 1996). The newly defined eosinophil subset of $CD11b^{hi}CD11c^-MHCII^-Ly6C^+Ly6G^-$ cells was found to express CD115, while inflammatory monocytes were negative (Figure 4.5). In addition, cells of the newly defined $CD11b^{hi}CD11c^{lo}Ly6C^+Ly6G^-$ subset were also $CD115^+$ as was the $Ly6C^+Ly6G^+$ L-DC candidate (Figure 4.5). CD115 expression did not further delineate resident monocytes and the $Ly6C^+Ly6G^-$ L-DC candidate subset, suggesting an overlap between the two populations. The absence of CD115 expression on inflammatory monocytes was unexpected, but

could be attributed to the use of a different antibody clone in these experiments, or to weaker expression of CD115 on splenic monocytes as opposed to blood monocytes. The inflammation-associated 7/4 antigen was also used to further delineate subsets of myeloid cells and L-DC candidates. In line with the literature, both neutrophils and inflammatory monocytes were positive for 7/4 (Henderson et al., 2003; Hirsch and Gordon, 1983; Taylor et al., 2003). Interestingly, both the $Ly6C^{+}Ly6G^{+}$ and $Ly6C^{+}Ly6G^{-}$ L-DC candidate subsets were also positive for 7/4, consistent with other evidence that both $Ly6C^{+}$ L-DC candidate subsets are contaminated with neutrophils and/or inflammatory monocytes.

Phenotypic studies were informative, but not definitive in identification of L-DC and myeloid subsets. Thus, morphological characterisation by May-Grünwald-Giemsa staining was used to confirm predictions. Blood monocytes have been described as cells with bi-lobate nuclei and minimal cytoplasm devoid of granules (Geissmann et al., 2003; Sunderkötter et al., 2004). Only inflammatory monocytes displayed this morphology in line with their blood counterparts (Swirski et al., 2009; Voehringer et al., 2007). Giemsa staining confirmed that the splenic $CD11b^{hi}CD11c^{-}$ $Ly6C^{+}Ly6G^{-}$ subset, newly categorised as eosinophils, had the characteristic multi-lobate nucleus with orange granules in the cytoplasm, consistent with their phenotypic classification as $Siglec\text{-}F^{+}$ eosinophils (Mori et al., 2009). The $Ly6C^{+}Ly6G^{-}$ L-DC candidate displayed morphology consistent with blood monocytes and inflammatory monocytes, but not with DC. Cells had a bi-lobate nucleus with minimal cytoplasm lacking granules. On the basis of phenotypic and morphological studies, it was concluded that spleen resident monocytes must lie within the $Ly6C^{+}Ly6G^{-}$ L-DC candidate population.

The morphology of splenic DC was found to be quite distinct from described Langerhans cells which have long membrane projections (Silberberg, 1973). $CD8^{+}$ cDC were found to be mononuclear cells with vacuoles evident in the cytoplasm. Both $CD8^{-}$ cDC and the majority of cells in the $Ly6C^{-}Ly6G^{-}$ L-DC candidate subset also demonstrated similar cDC morphology with vacuoles in the cytoplasm. By comparison, the $Ly6C^{+}Ly6G^{+}$ L-DC candidate population comprised mainly cells with multi-lobate nucleus resembling neutrophils, and this was confirmed by their 7/4 staining. On the basis of combined phenotypic and morphological studies, the

$Ly6C^{-}Ly6G^{-}$ L-DC candidate subset of $CD11b^{hi}CD11c^{lo}MHCII^{-}$ cells is most consistent with an *in vivo* equivalent of LTC-DC. It is also distinct from other splenic myeloid subsets and cDC based on phenotype, and consistent with a novel dendritic-like cell subset in spleen.

There have been at least four types of macrophages described within the spleen, each distinguishable by their anatomical location. These have mainly been identified immunohistologically using specific antibodies, but not extensively characterised by flow cytometry. Here the phenotype of splenic macrophages was analysed in relation to the splenic myeloid and DC subsets under investigation here using four distinct markers: CD68, MOMA-1, SIGNR1 and F4/80. The majority of marker positive cells had the phenotype of $CD11b^{+}CD11c^{-}Ly6C^{-}Ly6G^{-}$ cells (Figure 4.8). Some macrophages have been described as $CD11c^{+}$ cells, but CD11c expression appears to be an anatomically location-specific marker, e.g. it is expressed by pulmonary macrophages in particular (Gonzalez-Juarrero et al., 2003; Gower et al., 2011; Soos et al., 2006; Vallon-Eberhard et al., 2006). L-DC phenotypically resemble macrophages, since they lack MHCII expression and have high expression of CD11b. They have been shown here to be clearly distinct from splenic macrophages. Only low-level contamination (0.5-5%) of marker positive splenic macrophages was found amongst the myeloid, L-DC and cDC subsets under study here (Figures 4.9 - 4.12). Overall, L-DC lack expression of MOMA-1, SIGNR1 and CD68, distinguishing them from marginal-zone metallophilic macrophages, marginal-zone macrophages and white pulp macrophages. By contrast, F4/80 staining which defines red pulp macrophages (Austyn and Gordon, 1981; McKnight and Gordon, 1998; Morris et al., 1991), was expressed by cDC, L-DC and all myeloid subsets. Uniform expression of F4/80 across many splenic subsets could implicate it as marker of splenic red pulp location rather than a specific subset marker.

In conclusion, a novel dendritic-like cell type, L-DC, has been defined as a $CD11b^{hi}CD11c^{lo}Ly6C^{-}Ly6G^{-}MHCII^{-}$ subset resembling *in vitro* grown LTC-DC. L-DC are distinct from cDC due to lack of MHCII expression, and also distinct from known myeloid cells due to the absence of specific myeloid markers. In addition, splenic myeloid subsets have been redefined here by employing further markers and

morphological analysis. The $CD11b^{hi}CD11c^{-}Ly6C^{+}Ly6G^{-}$ phenotype described in the literature for resident monocytes in blood was found to identify eosinophils in spleen, while an alternative resident monocyte subset in spleen has been defined as $CD11b^{hi}CD11c^{lo}Ly6C^{+}Ly6G^{-}CD43^{+}CX_3CR1^{hi}CD115^{+}Siglec\text{-}F^{-}$ cells. While phenotype analysis is useful in identification of cells, it should not be the only benchmark for delineation. Thus, a combination of phenotypic, morphological, functional (Chapter 5) and genetic studies (Chapter 6) have been used to further identify subsets.

# Chapter 5

# Functional characterisation of L-DC in relation to splenic dendritic and myeloid cell subsets

## 5.1 Introduction

Dendritic cells (DC) play an important role in the immune system by acting as a mediator between the innate and adaptive immune responses (Heath et al., 2004). Under steady-state conditions, DC process and present self antigen to T cells to maintain self-tolerance and to prevent autoimmunity (Heath et al., 2004; Shortman and Naik, 2007). However, during infection and inflammation, autoimmunity is also kept in check by regulatory T (Treg) cells (Sakaguchi et al., 2008). During infectious states, DC become activated by danger signals and recruit other leukocytes to sites of infection leading to activation of the adaptive immune response (Shortman and Naik, 2007). Most importantly, DC that acquire infectious agents activate T cells and mount an antigen-specific immune response against the pathogen (Heath et al., 2004; Shortman and Naik, 2007). Since multiple DC subsets have been described, it is important to characterise them in terms of their function, and to identify their interaction with other leukocytes for better understanding of their role in the maintenance of tolerance and in inducing immune responses.

Professional antigen presenting cells (APC) can process and present exogenous antigen to $CD4^+$ T cells as peptides on MHCII molecules. However, some splenic DC have a unique ability to cross-present antigen to $CD8^+$ T cells (Shortman and Heath, 2010). Exogenous antigens are processed and presented as peptides bound to MHCI molecules for $CD8^+$ T cell activation. Cross-presentation can occur by two pathways: the cytosolic and vacuolar pathways (Heath and Carbone, 2001). The cytosolic pathway involves uptake of exogenous antigen by endosomes with subsequent release into the cytoplasm where it is degraded by proteasomes. Peptides are then transported into the endoplasmic reticulum (ER) by transporters associated with antigen processing, the TAP molecules (Heath and Carbone, 2001). Subsequently, peptides are loaded on to MHCI molecules which are then shuttled to the cell membrane for presentation to $CD8^+$ T cells. While this is the most likely mechanism for cross-presentation, there has been no direct evidence that the loading of peptides on to MHCI molecules occurs in the ER. However, TAP and the MHCI loading complex have been described in phagosomes and endosomes (Burgdorf et al., 2008; Monu and Trombetta, 2007), so that it is likely that the loading of peptide on to MHCI can occur in the cytoplasm. The vacuolar pathway is distinct from the

cytosolic pathway such that antigen processing occurs within endosomes (Heath and Carbone, 2001). These contain lysosomes that break antigen down into peptides. The loading of peptide on to MHCI occurs when vesicles carrying MHCI molecules fuse with peptide-rich endosomes. The use of these pathways by DC is still not well defined. However, it is possible that DC can use both pathways under different conditions, and there is evidence in the literature which supports this possibility (Joffre et al., 2012).

LTC-DC are capable of cross-presenting antigen to $CD8^+$ T cells (O'Neill et al., 2011; O'Neill et al., 2004; Quah et al., 2004; Wilson et al., 2000) (Chapter 3). Their cross-presenting ability, as well as their high endocytic capacity, can therefore be used as defining properties in the identification of a similar cell type *in vivo*. In Chapter 3, the ability of a $CD11b^{hi}CD11c^{lo}MHCII^-CD8^-$ L-DC subset to cross-prime $CD8^+$ T cells was determined in an *in vitro* assay by pulsing the sorted L-DC subset with OVA prior to addition of labelled $CD8^+$ T cells from OT-I TCR-tg (anti-$H2K^b/OVA_{257-264}$) mice. However, the assay outcome was limited by low numbers of available L-DC. Conventional(c) DC are rare cells representing about one percent of spleen, and L-DC have an even lower frequency in spleen than cDC (Griffiths et al., 2013). In addition, antigen pulsing and washing steps used in *in vitro* assays also contribute to cell loss. To overcome the limitations of the *in vitro* assay for functional studies, Actm-OVA transgenic mice were employed here as a source of APC (Ehst et al., 2003). These mice constitutively express membrane-bound OVA under the actin promoter. Cross-presentation of antigen occurs *in vivo*, when APC endocytose dead cells and cell debris. However, this mouse model expresses high levels of cell-associated OVA protein. It is therefore possible that some OVA enters the endogenous antigen processing pathway within cells. For example, defective OVA could be tagged for ubiquitin destruction in the cytoplasm, entering proteasomes, and then be transported into the ER. For this reason, it was necessary to use control APC subsets which do not cross-present in order to gauge any background effect due to antigen overload. Since myeloid subsets have been described as unable to cross-prime $CD8^+$ T cells, these were used as controls in cross-presentation assays involving APC derived from Actm-OVA mice.

This chapter focuses on the functional characteristics of cDC and other myeloid subsets identified in Chapter 4, as well as the 'candidate' L-DC subsets under investigation. Five aspects of APC function have been investigated. These include the ability to uptake and retain antigen, to present antigen to $CD4^+$ T cells, to cross-present antigen to $CD8^+$ T cells, and to induce cytotoxic T lymphocytes.

## 5.2 Results

### 5.2.1 Comparison of the endocytic capacity of L-DC, cDC and myeloid cells

Spleen is a secondary lymphoid organ that is specialised in filtering blood-borne antigens. It is anticipated therefore that some splenic dendritic and myeloid cells are readily able to endocytose antigen. In this study, pinocytosis and receptor-mediated endocytosis of antigen were investigated. Previous studies showed that LTC-DC are highly endocytic (O'Neill et al., 2004; Tan et al., 2011), and so this property was also used to distinguish L-DC candidates. Soluble OVA conjugated with FITC (OVA-FITC) was delivered intravenously to C57BL/6J mice at different times (1, 3 and 6 hours) prior to euthanasia. Spleens were collected for analysis and flow cytometric assessment of uptake of OVA-FITC in different splenic dendritic and myeloid subsets was performed as described in Section 2.5.1. Consistent with the literature, both $CD8^+$ cDC and $CD8^-$ cDC demonstrated ability to endocytose and retain antigen over a 3 hour period (Figure 5.1 A). $CD8^+$ cDC demonstrated some ability to retain antigen, since 11.2% of cells retained antigen after 6 hours (Figure 5.1 A).

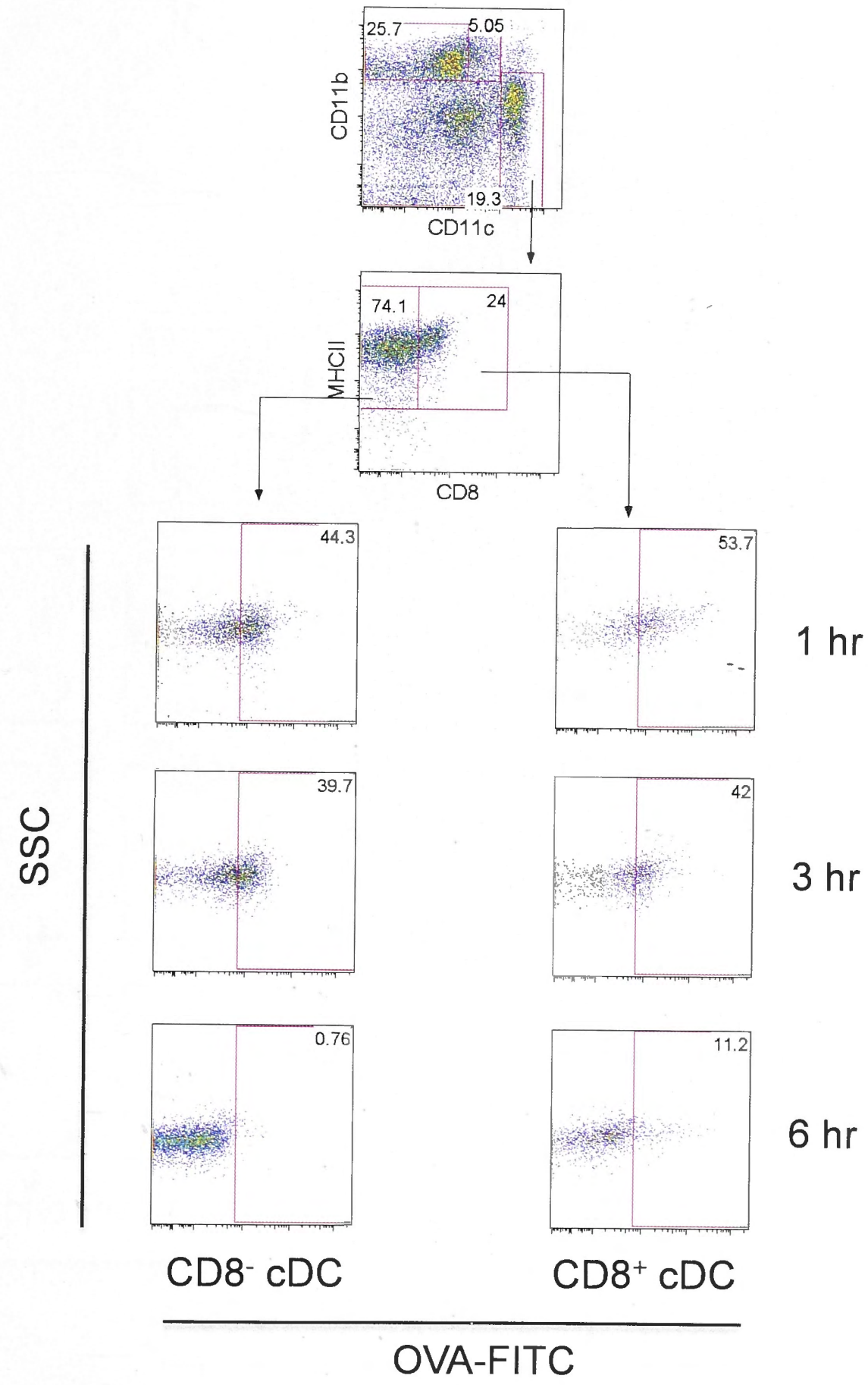
Among the myeloid subsets, both resident and inflammatory monocytes showed ability to endocytose and retain antigen, with resident monocytes the most potent. Only ~9% of neutrophils and eosinophils took up and retained antigens after 6 hours. Their endocytic capacity was relatively weak compared with the monocyte and cDC subsets (Figure 5.1 A and B). L-DC displayed ability to endocytose antigen, but the level of uptake was relatively low compared with monocytes (Figure 5.1 A and B). Overall, resident monocytes showed the strongest ability to endocytose and retain antigen over a 6 hour period, followed by $CD8^+$ cDC, $CD8^-$ cDC, inflammatory monocytes, L-DC, then neutrophils and eosinophils (Figure 5.1 C).

In terms of receptor-mediated endocytosis, different cell types can have a unique combination of receptors on their cell membrane to aid uptake of antigen in various forms. Mannose receptor-mediated uptake of antigen in DC has been shown to play a role in cross-presentation of antigen to $CD8^+$ T cells (Burgdorf et al., 2007; Burgdorf et al., 2006). Cross-presentation is a defining property of DC, especially

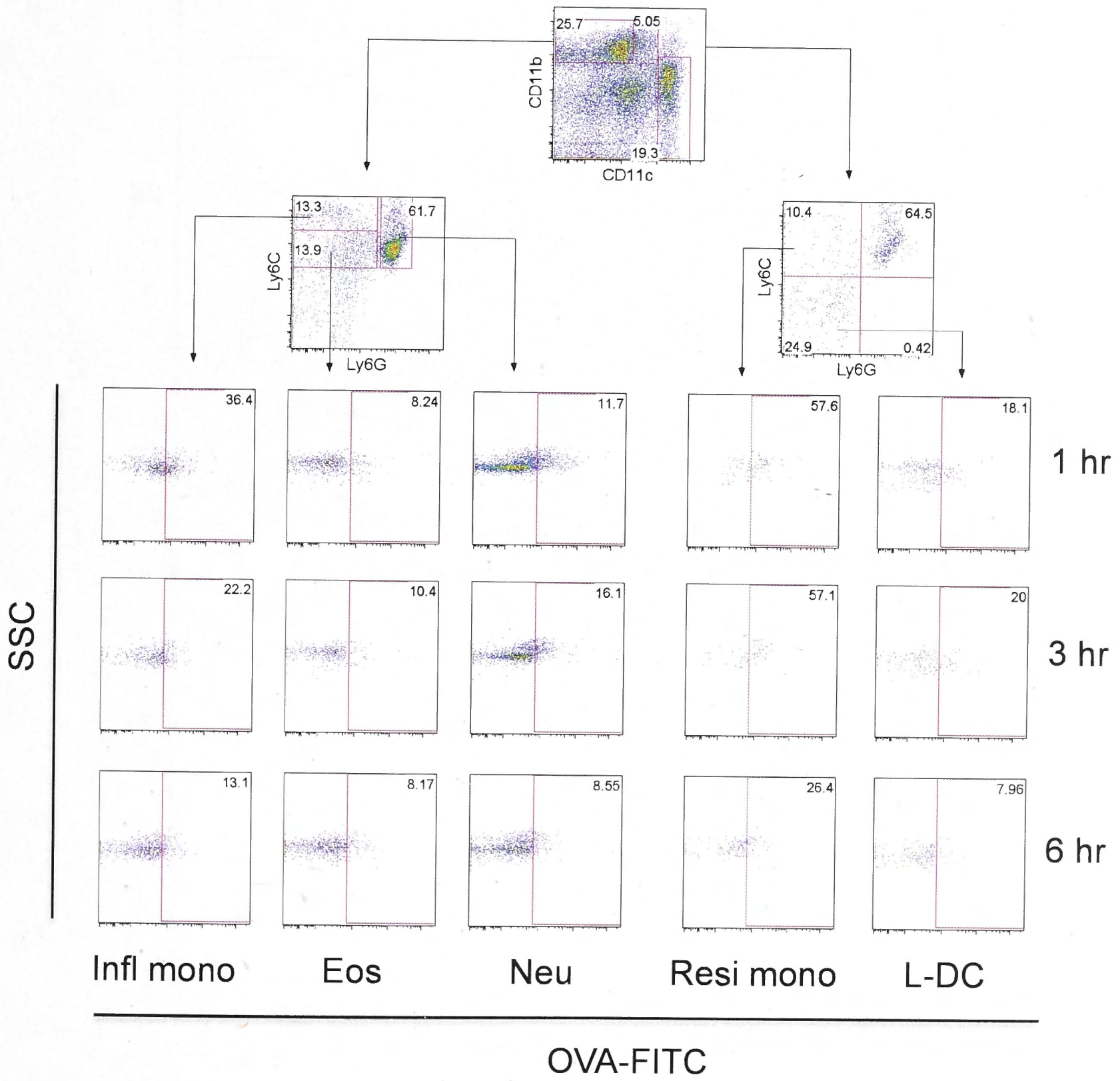
**Figure 5.1 Endocytic ability of myeloid and DC subsets.**

The ability of cells to endocytose antigen was measured by uptake of FITC-conjugated ovalbumin (OVA-FITC). C57BL/6J mice were given OVA-FITC intravenously (i.v.; 1mg per mouse) at times of 1, 3 and 6 hours prior to euthanasia for spleen collection. Control mice were given PBS. Spleen cells were RBC lysed and enriched for dendritic and myeloid cells via T and B cell depletion. Cells were stained with 2 distinct antibody cocktails. The first included antibodies to CD11b (M1/70, PE-Cy7), CD11c (N418, APC), Ly6C (Al-21, PE) and Ly6G (1A8, Biotin). The second contained antibodies to CD11b (M1/70, PE-Cy7), CD11c (N418, APC), CD8 (53-6.7, PE) and MHC-II (25-9-17, Biotin). Streptavidin (SA)-APC-Cy7 was used as a second stage reagent. Prior to flow cytometry, cells were stained with propidium iodide (PI, 1μg/ml) to delineate live ($PI^-$) cells. A) cDC were gated as $CD11b^{+/-}CD11c^{hi}$ cells and further delineated to give $CD8^+$ cDC ($CD8^+MHC\text{-}II^+$) and $CD8^-$ cDC ($CD8^-MHC\text{-}II^+$) for assessment of OVA-FITC staining. B) Myeloid cells were gated as a $CD11b^{hi}CD11c^-$ subset, and further delineated to give eosinophils (Eos : $Ly6C^+Ly6G^-$), inflammatory monocytes (Infl mono : $Ly6C^{hi}Ly6G^-$) and neutrophils (Neu : $Ly6C^+Ly6G^+$). Resident monocytes (Resi mono) were gated as $CD11b^{hi}CD11c^{lo}Ly6C^+Ly6G^-$ cells, while L-DC were gated as $CD11b^{hi}CD11c^{lo}Ly6C^-Ly6G^-$ cells. Uptake of OVA-FITC was assessed as % FITC staining cells. Gates were set based on fluorescence minus one controls. Numbers in quadrants reflect % positive cells. C) Time course of OVA-FITC uptake by cells.

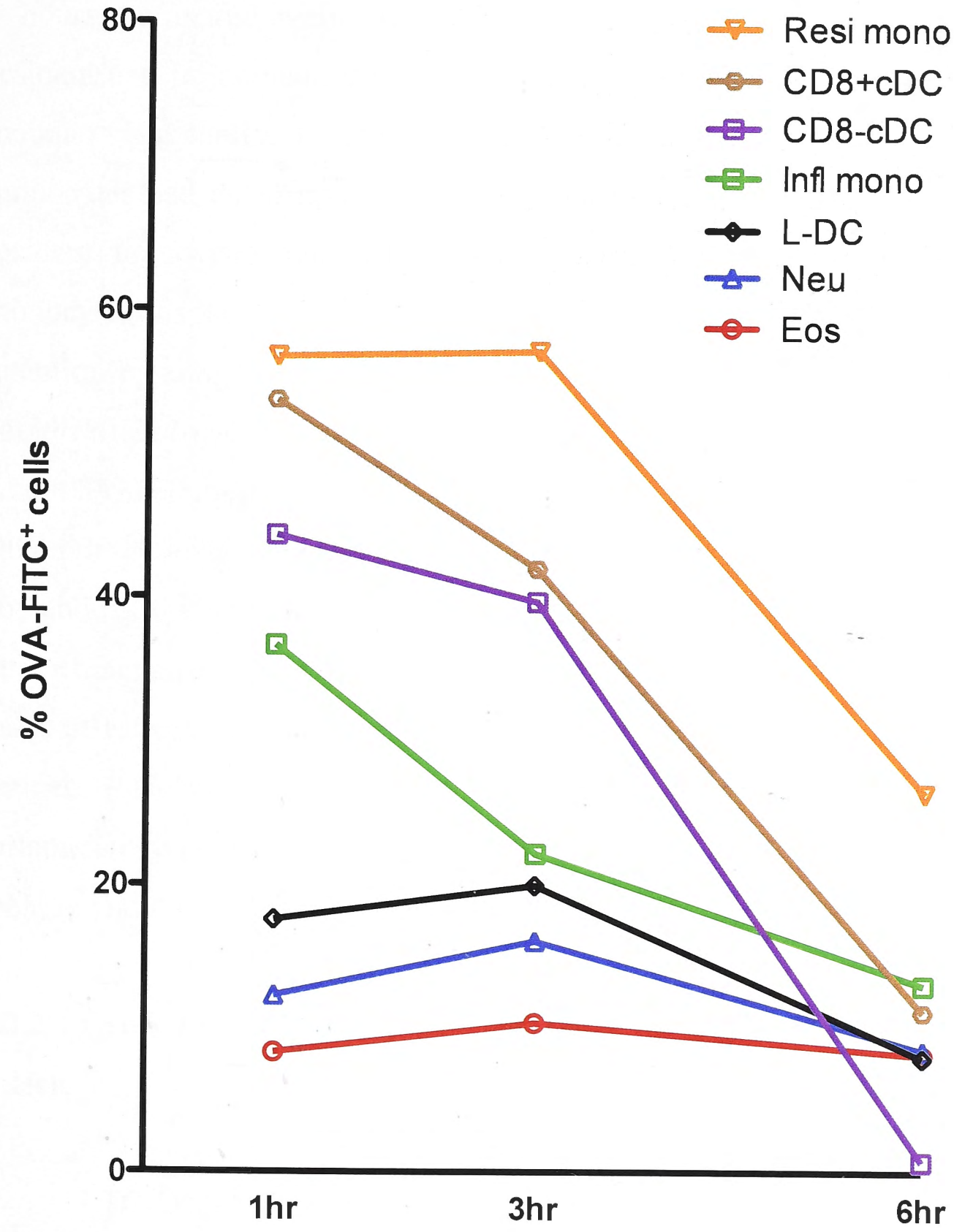
A


25.7
5.05
19.3
CD11b
CD11c
74.1
24
MHCII
CD8
44.3
53.7
1 hr
39.7
42
3 hr
0.76
11.2
6 hr
SSC
CD8- cDC
CD8+ cDC
OVA-FITC

B


25.7
5.05
19.3
CD11b
CD11c
13.3
61.7
13.9
Ly6C
Ly6G
10.4
64.5
24.9
0.42
Ly6C
Ly6G
36.4
8.24
11.7
57.6
18.1
1 hr
22.2
10.4
16.1
57.1
20
3 hr
13.1
8.17
8.55
26.4
7.96
6 hr
SSC
Infl mono
Eos
Neu
Resi mono
L-DC
OVA-FITC

C


80
60
40
20
0
% OVA-FITC+ cells
1hr
3hr
6hr
Time post exposure
Resi mono
CD8+cDC
CD8-cDC
Infl mono
L-DC
Neu
Eos

splenic $CD8^+$ cDC (Heath et al., 2004). In order to determine if L-DC and other myeloid subsets could endocytose antigen via mannose receptor as do $CD8^+$ cDC, mannan-conjugated to FITC (mannan-FITC) was delivered intravenously to C57BL/6J mice and uptake monitored over time. Consistent with the literature, both $CD8^+$ cDC and $CD8^-$ cDC demonstrated strong ability to take up antigen via the mannose receptor. Over 50% of $CD8^-$ cDC took up mannan-FITC and retained it for 6 hours, compared with ~40% of $CD8^+$ cDC (Figure 5.2 A). Interestingly, both resident and inflammatory monocytes demonstrated the strongest ability to take up mannan via mannose receptor. By 1 hour after antigen delivery, over 75% of resident monocytes had taken up and retained mannan. Even after 6 hours, over half of the resident monocytes retained mannan (Figure 5.2 A). In contrast, inflammatory monocytes displayed peak uptake (88%) at 3 hours post delivery of mannan with retention of antigen for 6 hours by ~45% of cells (Figure 5.2 A). Delayed uptake could reflect lower accessibility to antigen in comparison with resident monocytes. About 35% of eosinophils showed ability to take up mannan at 3 hours post delivery, although the level of mannan diminished by 6 hours (Figure 5.2 A). Neutrophils did not endocytose mannan-FITC. L-DC showed similar high ability to take up and retain mannan as did $CD8^+$ cDC (Figure 5.2 A). In this *in vivo* assay, uptake of mannan reflects both cell ability for endocytosis and accessibility to blood-borne antigen. Resident monocytes took up and retained the most mannan, followed by inflammatory monocytes (Figure 5.2 B). Each of $CD8^+$ cDC, L-DC and $CD8^-$ cDC took up and retained mannan-FITC at similar levels (Figure 5.2 B).

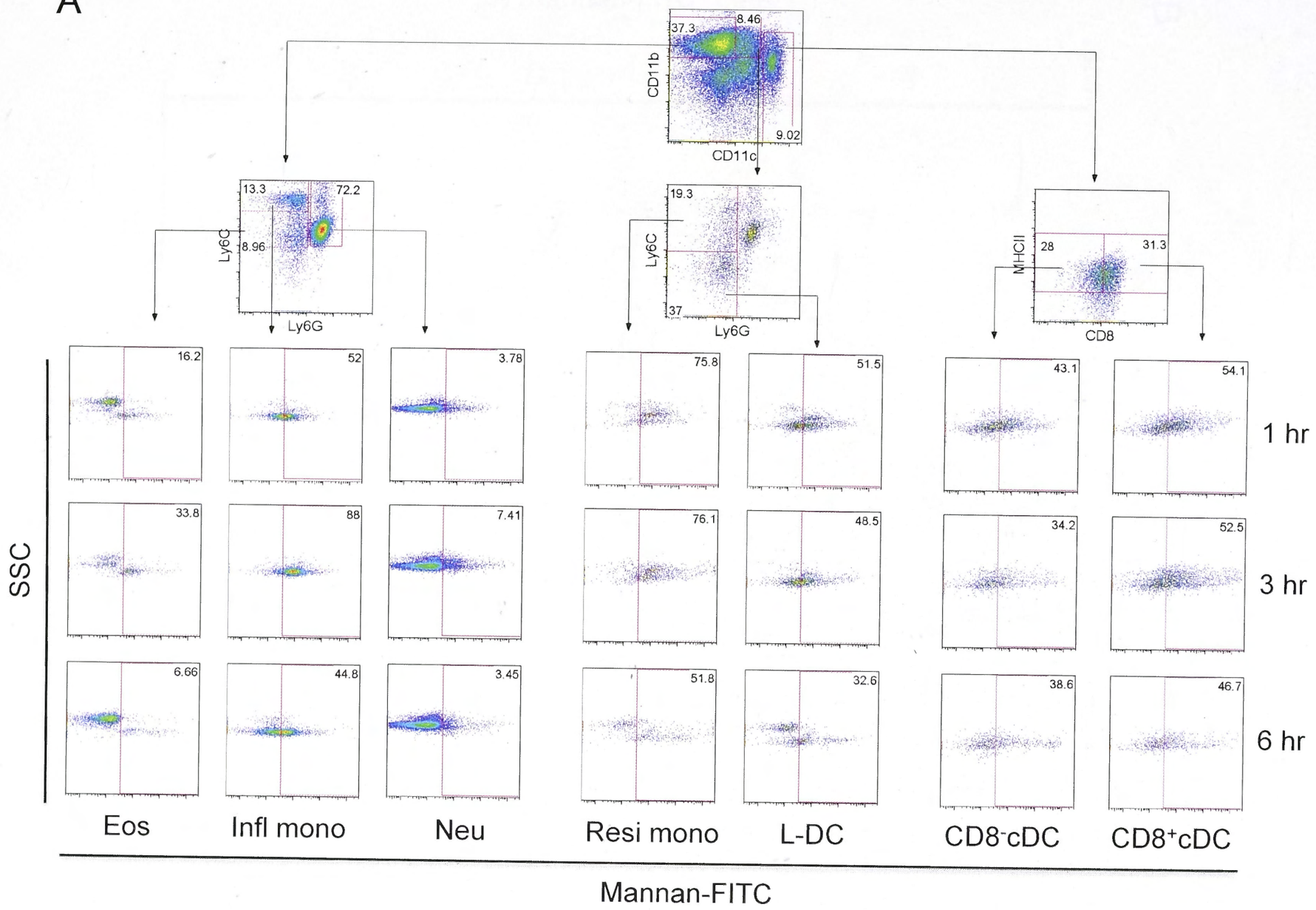
### 5.2.2 Cross-priming ability as a criterion for delineation of L-DC subsets in spleen

LTC-DC are known to be capable of cross-priming $CD8^+$ T cells (Tan et al., 2011). Therefore, cross-priming ability was used as a selection criterion for delineation of the *in vivo* equivalent of LTC-DC. This assay was therefore employed to determine if the $Ly6C^-Ly6G^-$ L-DC candidate delineated in Chapter 4 is in fact an *in vivo* equivalent of LTC-DC in terms of cross-presentation capacity. Splenic dendritic and myeloid subsets were therefore sorted from mACT-OVA mice according to the sorting plan used in Figure 4.3. The cross-priming ability of L-DC and other myeloid subsets for OT-I TCR-tg $CD8^+$ T cells was assessed and compared

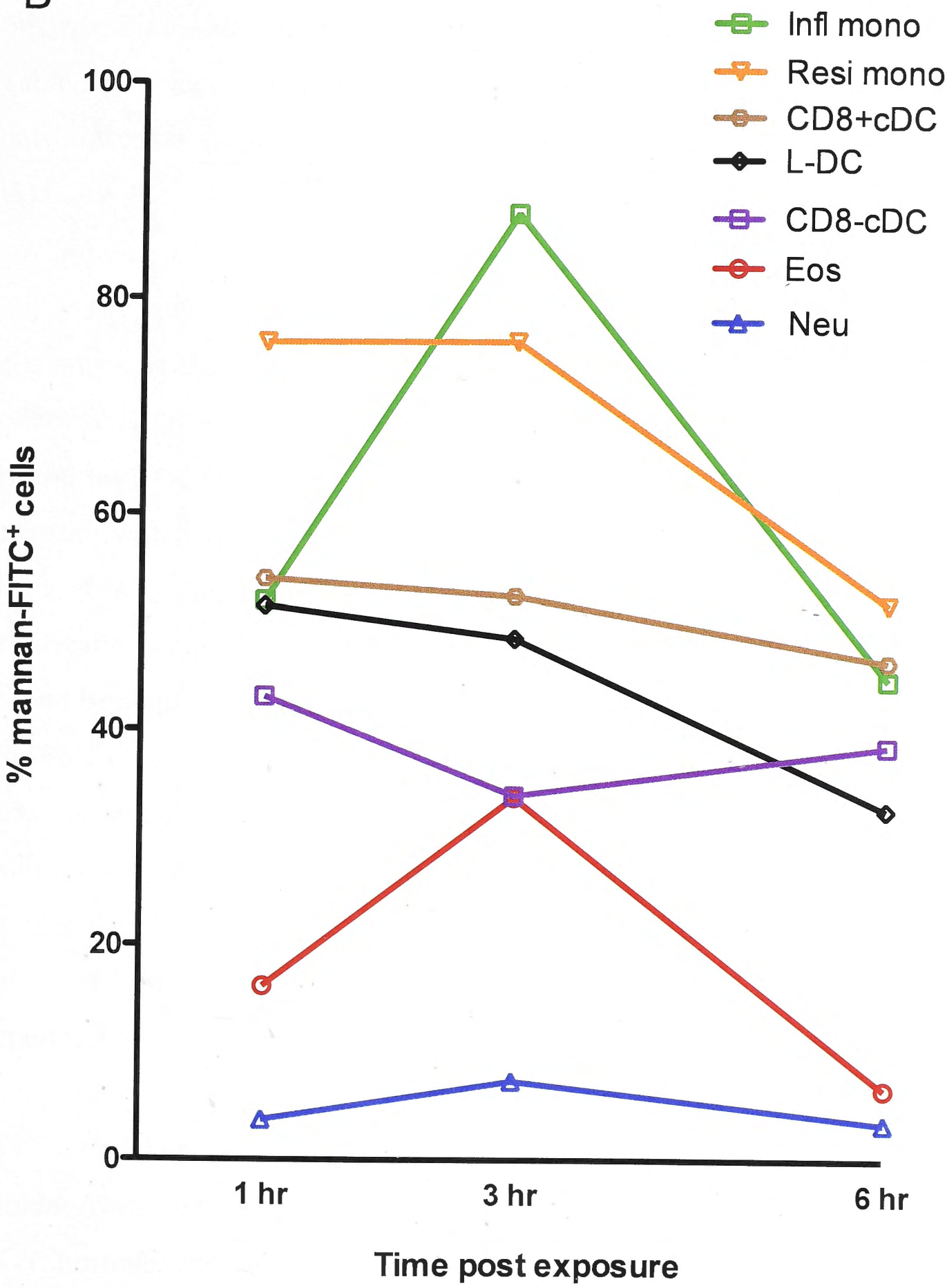
**Figure 5.2 Role of mannose receptor in antigen uptake.**

Ability of dendritic and myeloid subsets to take up antigen via mannose receptors was assessed in terms of uptake of FITC-conjugated mannan (mannan-FITC). This was given to C57BL/6J mice intravenously (i.v: 0.5mg per mouse) at 1, 3 and 6 hours before euthanasia for spleen collection. Control mice were given PBS. Spleen cells were prepared and stained for flow cytometry as described in the legend to Figure 5.1. Prior to flow cytometry, cells were stained with propidium iodide (PI, 1μg/ml) to delineate live ($PI^-$) cells. A) Myeloid cells were gated as $CD11b^{hi}CD11c^-$ cells and further delineated to give eosinophils (Eos: $Ly6C^+Ly6G^-$), inflammatory monocytes (Infl mono: $Ly6C^{hi}Ly6G^-$) and neutrophils (Neu: $Ly6C^+Ly6G^+$). Resident monocytes (Resi mono) were gated as $CD11b^{hi}CD11c^{lo}Ly6C^+Ly6G^-$ cells, while L-DC were gated as $CD11b^{hi}CD11c^{lo}Ly6C^-Ly6G^-$ cells. Conventional DC were gated as $CD11b^{+/-}CD11c^{hi}$ cells, and further delineated to give $CD8^+$ cDC ($CD8^+MHCII^+$) and $CD8^-$ cDC ($CD8^-MHCII^+$) subset. Uptake of mannan-FITC via mannose receptors was assessed as % mannan-FITC positive cells. Gates were set based on fluorescence minus one controls. Numbers in quadrants reflect % positive cells. B) Uptake of FITC-mannan by cells at over time.

A


CD11b
CD11c
37.3
8.46
9.02
Ly6C
Ly6G
13.3
72.2
8.96
19.3
37
MHCII
CD8
28
31.3
16.2
52
3.78
75.8
51.5
43.1
54.1
33.8
88
7.41
76.1
48.5
34.2
52.5
6.66
44.8
3.45
51.8
32.6
38.6
46.7
1 hr
3 hr
6 hr
SSC
Eos
Infl mono
Neu
Resi mono
L-DC
CD8⁻cDC
CD8⁺cDC
Mannan-FITC

B


Infl mono
Resi mono
CD8+cDC
L-DC
CD8-cDC
Eos
Neu
100
80
60
40
20
0
% mannan-FITC+ cells
1 hr
3 hr
6 hr
Time post exposure

with that of $CD8^+$ cDC and $CD8^-$ cDC subsets, which serve as positive controls. The assay was performed in the presence and absence of lipopolysaccharide (LPS) which acts as a potent inflammatory stimulus for some DC (Akira and Takeda, 2004). Across experiments I, II and III (Figure 5.3), both $CD8^+$ cDC and $CD8^-$ cDC showed strong ability to cross-prime $CD8^+$ OT-I T cells, with $CD8^-$ cDC marginally stronger than $CD8^+$ cDC. L-DC demonstrated weaker ability to cross-prime $CD8^+$ OT-I T cells requiring ~30 fold more L-DC to induce an equivalent response as $CD8^-$ cDC (Table 5.1). Neutrophils in Experiment I, and resident monocytes in Experiment II, showed weaker ability to induce proliferation in $CD8^+$ T cells than did L-DC (Figure 5.3).

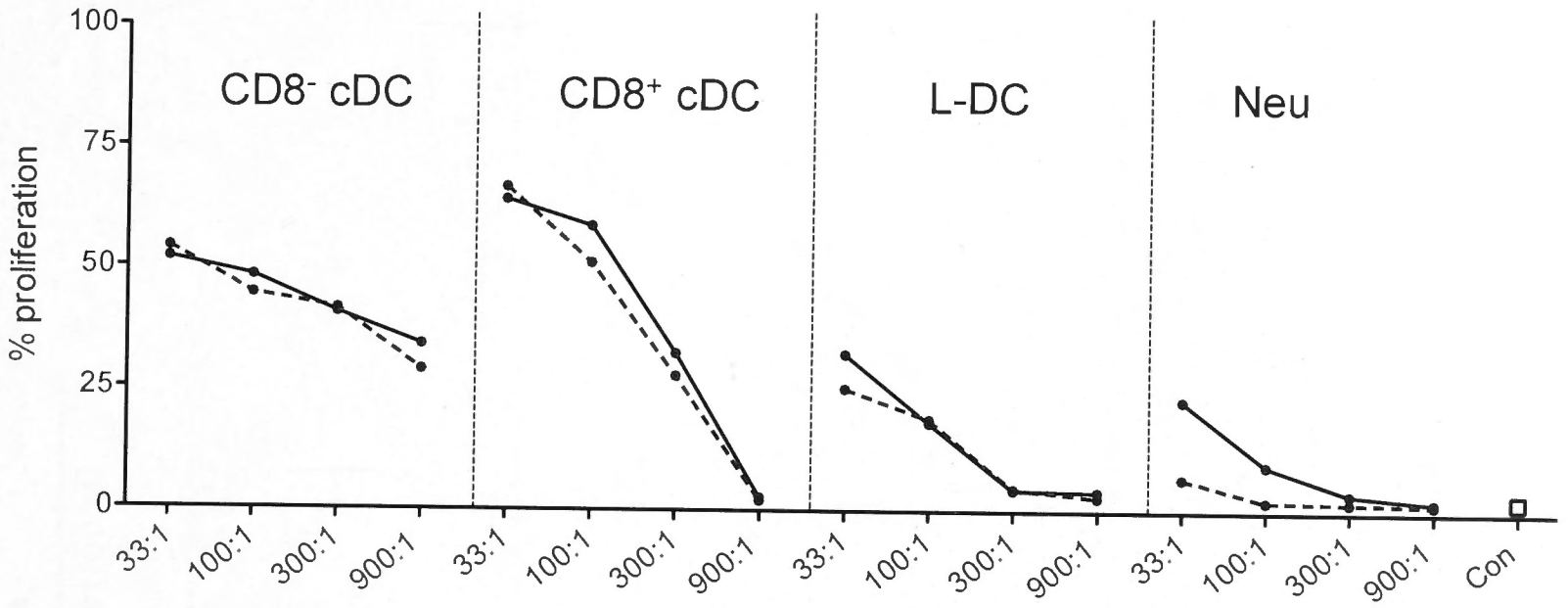
The ratio of T cells/APC required to induce 50% proliferation of $CD8^+$ T cells was calculated in order to compare data across experiments (Table 5.1). This collective data showed that isolated $CD8^-$ cDC demonstrated the strongest ability to cross-prime $CD8^+$ T cells, even above $CD8^+$ cDC. This is contradictory to the literature which compares isolated antigen-pulsed $CD8^+$ cDC and $CD8^-$ cDC (Den Haan et al., 2000; Pooley et al., 2001). $CD8^-$ cDC induced 50% maximum proliferation of $CD8^+$ T cells at 900 T cells/APC (Table 5.1). $CD8^+$ cDC were the second best inducer of proliferation in $CD8^+$ T cells, requiring more APC at a ratio of ~300:1 T cells/APC (Table 5.1). L-DC were required at a higher number and induced 50% maximum proliferation at ratios of 33 or 100 $CD8^+$ T cells/L-DC (Table 5.1). Both resident monocytes and neutrophils showed weaker ability to cross-prime $CD8^+$ T cells than L-DC (Table 5.1). In addition, the presence of LPS added into co-cultures in this protocol did not improve the outcome of T cell activation across 5 experiments for all cell types tested (I to V) (Table 5.1).

The cross-presenting ability of myeloid subsets in relation to DC has been the subject of debate. Initial studies suggested that cross-presentation was restricted to DC (Shortman and Heath, 2010). However, some macrophages and neutrophils have been described as having cross-presenting ability under various conditions (Beauvillain et al., 2007; Harding and Song, 1994; Kovacsovics-Bankowski et al., 1993; Norbury et al., 1995; Pfeifer et al., 1993). Splenic myeloid subsets sorted from mACT-OVA mice were therefore compared with the candidate L-DC subset for ability to cross-present OVA, with the aim of further delineating the L-DC subset

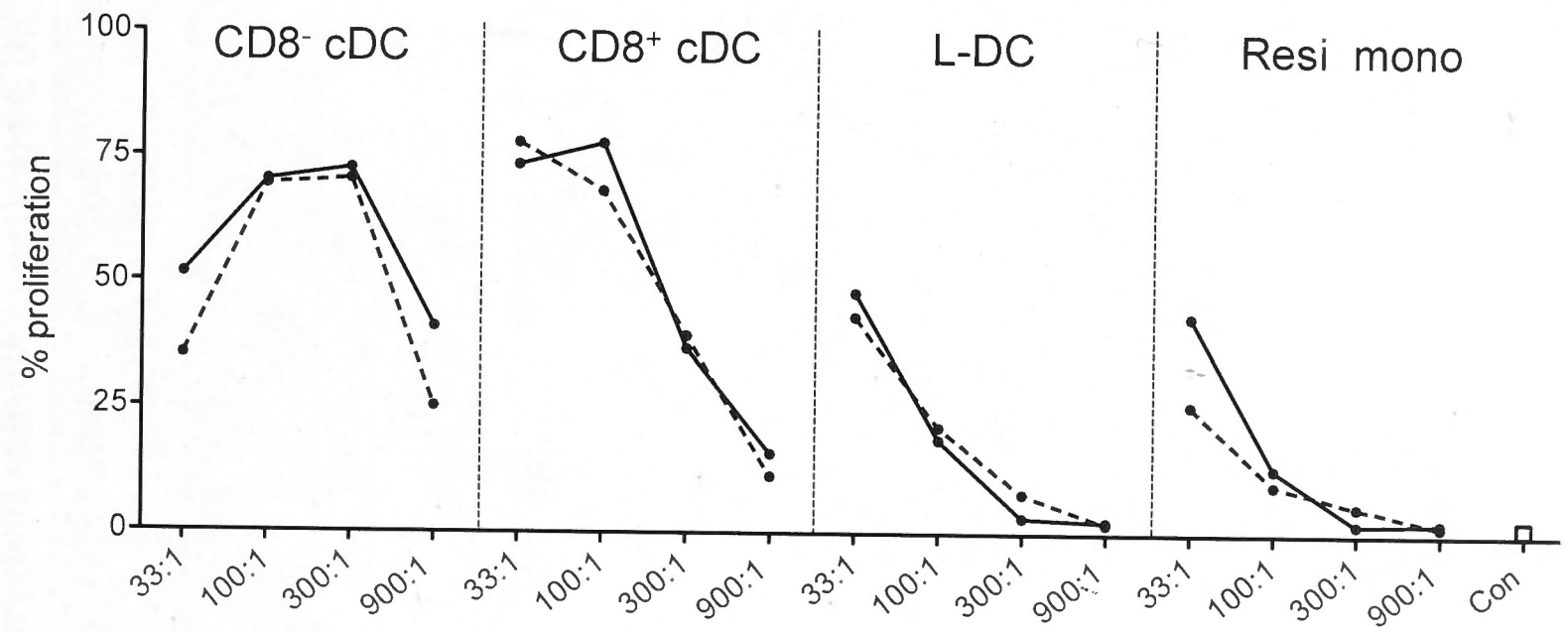
**Figure 5.3 Cross priming capability of dendritic and myeloid cell subsets.**

Splenocytes were harvested from Act-mOVA mice and prepared by red blood cell lysis and T/B cell depletion. In experiments I, II and III, enriched splenocytes were stained with CD11b (M1/70, PE-Cy7), CD11c (N418, APC), Ly6C (AL-21, FITC), Ly6G (1A8, PE) and MHCII (25-9-17, Bio), using streptavidin (SA)-APC-Cy7 as a second stage reagent. Cells were sorted as described in Figure 3.2. Conventional DC were gated as $CD11c^{hi}MHCII^{+}Ly6C^{-}Ly6G^{-}$ cells and further delineated to give $CD8^{+}$cDC and $CD8^{-}$ cDC on the basis of CD11b expression. L-DC were gated as $CD11b^{hi}CD11c^{lo}Ly6C^{-}Ly6G^{-}MHCII^{-}$ cells. In experiment I, neutrophils (Neu) were gated as $CD11b^{hi}CD11c^{-}Ly6C^{+}Ly6G^{+}MHCII^{-}$ cells. In experiment II, resident monocytes (resi mono) were gated as $CD11b^{hi}CD11c^{lo}Ly6C^{+}Ly6G^{-}MHCII^{-}$ cells. Diluting numbers of APC were plated followed by treatment with or without lipopolysaccharide (LPS: 10ug/ml) for 2 hours prior to the addition of $10^{5}$ CFSE-labelled OT-I (TCR-tg) $CD8^{+}$ T cells. The latter were purified from OT-I mouse spleen through depletion of B cells, $CD4^{+}$ T cells, DC and myeloid cells using magnetic bead protocols. In experiment III, OT-I $CD8^{+}$ T cells were sorted from OT-I mice as $PI^{-}Thy1.2^{+}V\alpha2^{+}CD4^{-}$ cells. Cells were cocultured with APC in T cell : APC ratios of 33:1, 100:1, 300:1 and 900:1. After 72 hours, $CD8^{+}$ OT-I T cells were gated as $PI^{-}CD11b^{-}Thy1.2^{+}V\alpha2^{+}$ cells, and assessed flow cytometrically for CFSE dilution as an indicator of proliferation. OT-I T cells alone also served as control (con). Graphs show % proliferation of OT-I cells.

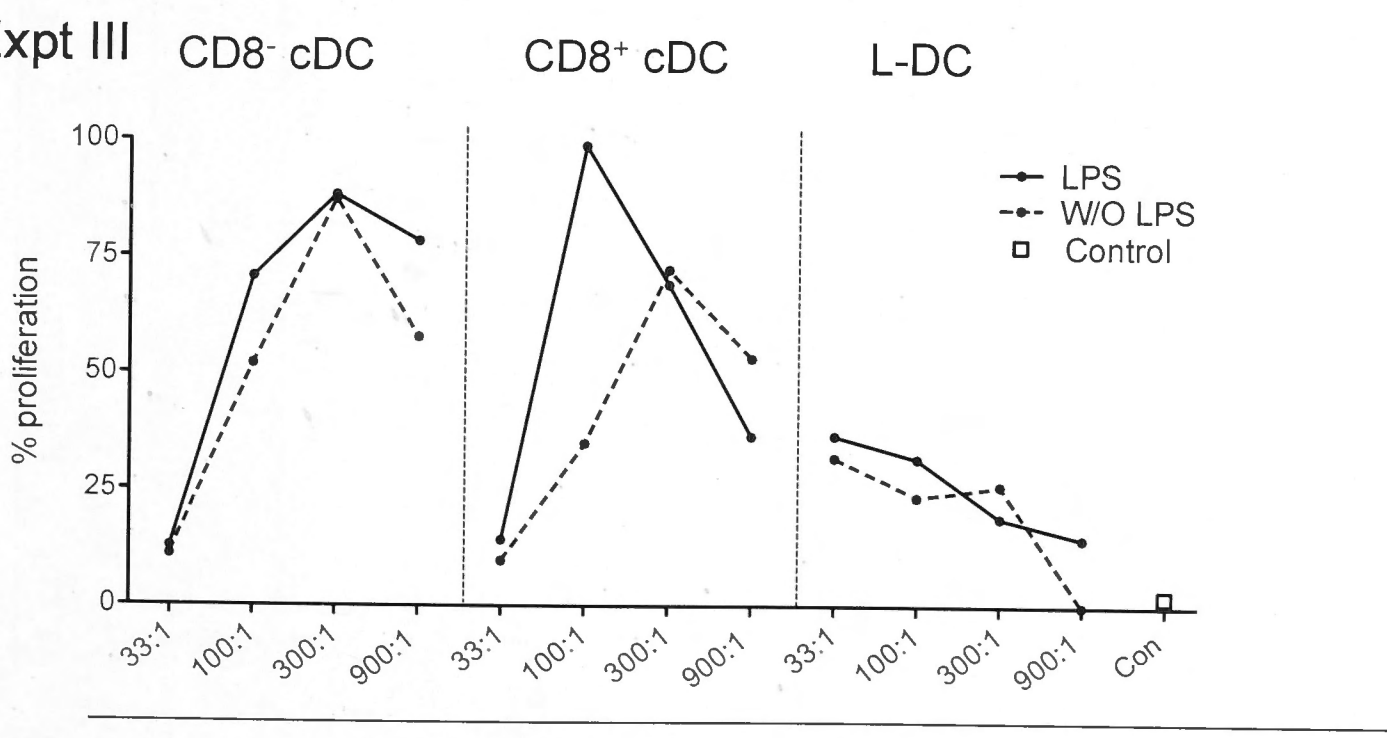
Expt I




Expt II




Expt III




T cell : APC ratio

**Table 5.1 Ability of dendritic and myeloid subsets to activate $CD8^+$ T cells.**

| Expt | Condition | T cell/APC ratio giving 50% maximum proliferation of OT-I T cells | | | | |
|---|---|---|---|---|---|---|
| | | $CD8^-$ cDC | $CD8^+$ cDC | L-DC | Resi Mono[a] | Neu[b] |
| **I** | + LPS | 900 | 300 | 33 | - | 33 |
| | - LPS | 900 | 300 | 33 | - | << 33 |
| **II** | + LPS | 900 | 300 | 33 | 33 | - |
| | - LPS | ~ 900 | 300 | 33 | < 33 | - |
| **III** | + LPS | > 900 | 900 | 33 | - | - |
| | - LPS | > 900 | 900 | 33 | - | - |
| **IV** | + LPS | > 900 | 300 | 100 | 42 | - |
| | - LPS | > 900 | 141 | 100 | 42 | - |
| **V** | + LPS | > 900 | > 900 | 100 | 100 | - |
| | - LPS | > 900 | > 900 | 100 | 100 | - |

[a] Resi mono : Resident monocytes [b] Neu : Neutrophils

from myeloid cells. Both resident and inflammatory monocytes showed some ability to cross-prime $CD8^+$ T cells, although resident monocytes gave a stronger $CD8^+$ OT-I T cell response (Figure 5.4). The T cell/APC ratio which gave 50% maximum proliferation, was also tabulated for experiments VI – VIII (Table 5.2). The presence of LPS induced a marginal increase in cross-priming response for L-DC under this protocol, while other APC remained unaffected (Table 5.2). While outcomes from different experiments varied slightly, there was an overall trend showing that L-DC were similar to the $CD43^+$ subset of cDC, but were better APC than resident monocytes, inflammatory monocytes and neutrophils.

### 5.2.3 The effect of cytochrome c on cross-presentation capacity

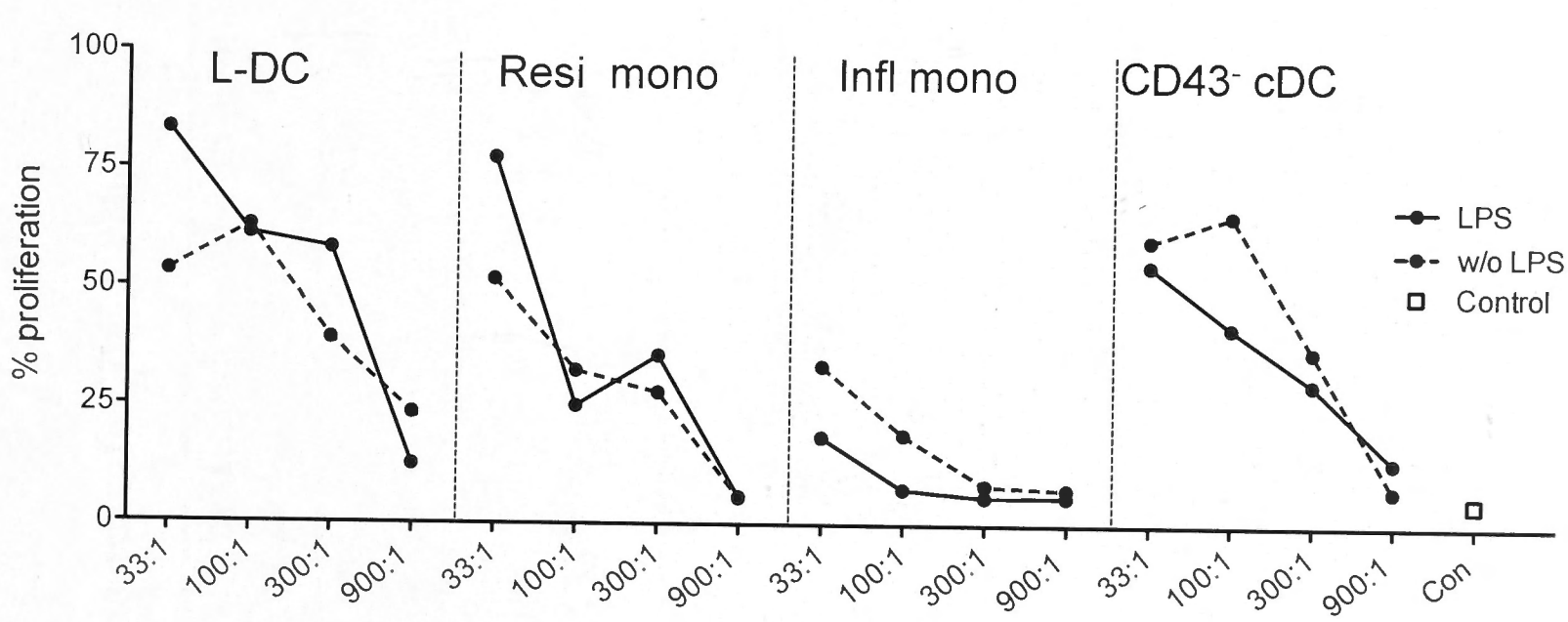
Uptake of cytochrome c via endocytosis, and entry into the cytoplasm via cross-presentation, can lead to cell death (Ming et al., 2008). Since cytochrome c selectively kills $CD8^+$ cDC, this suggests that the mechanism for cross-presentation by $CD8^+$ cDC involves the cytosolic pathway (Ming et al., 2008). L-DC were therefore tested to determine if cytochrome c treatment leads to cell death, indicative of cross-presentation via the cytosolic pathway. Cytochrome c was injected intravenously into C57BL/6J mice, and changes in the representation of all dendritic and myeloid subsets determined after 6 hours (Figure 5.5). No difference was found in the size of the treated populations compared with controls for $CD8^+$ cDC, $CD8^-$ cDC, L-DC, neutrophils or inflammatory monocytes (Student's t test: $p \leq 0.01$). Further analysis of treatment on $CD8^-$ cDC involved the Wilcoxon test since the variance of the treated and control populations was different. This test again showed no significant difference at $p \leq 0.05$. A significant increase in the subset size of resident monocytes was observed (Student's t test: $p \leq 0.01$), while a significant decrease in % eosinophils was observed (Student's t test: $p \leq 0.01$). Since an increase in cell number is unlikely to be due to the direct effect of cytochrome c on cells, this change in resident monocytes could reflect a population change due to treatment *in vivo*.

To further determine the effect of cytochrome c on the cross-presenting capacity of splenic APC, sorted cells from mACT-OVA mice were treated with cytochrome c prior to co-culture with CFSE-labelled OT-I $CD8^+$ T cells for

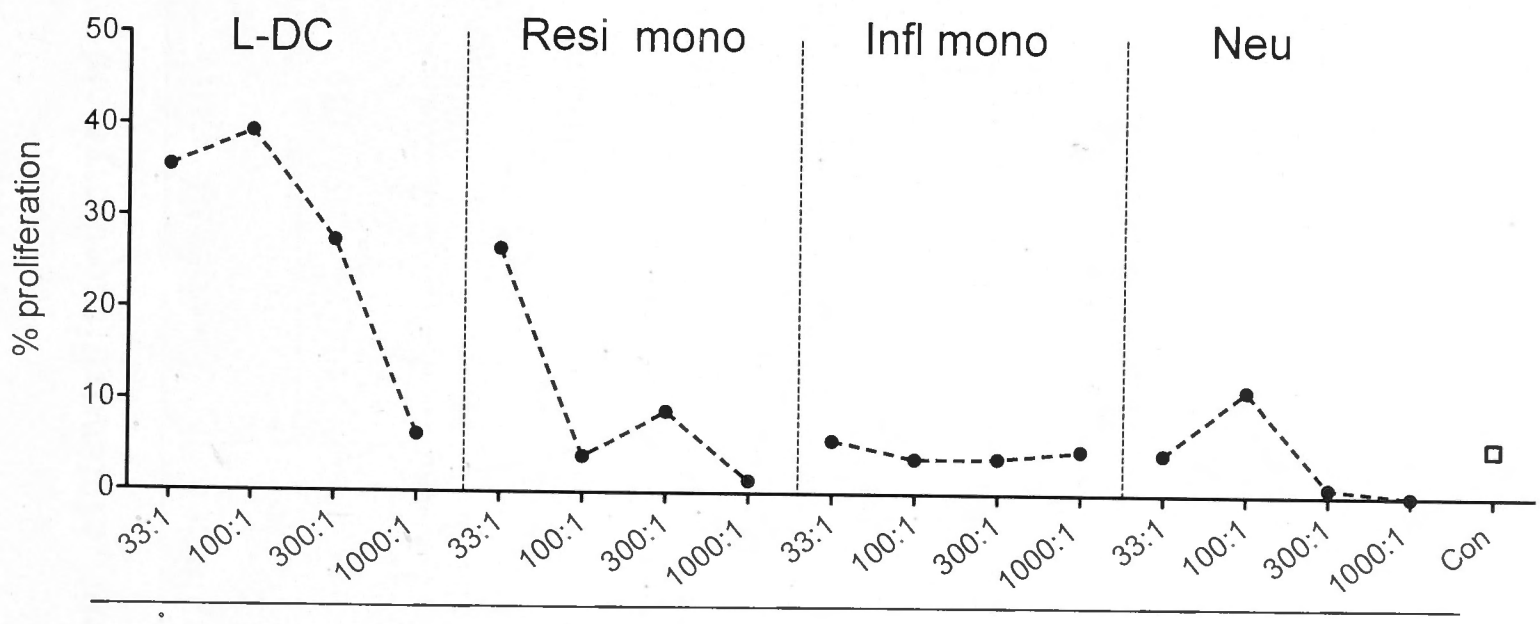
**Figure 5.4 Cross priming capability of L-DC compared with monocyte subsets.**

Splenocytes were harvested from Act-mOVA mice and prepared by red blood cell lysis and T/B cell depletion. In experiments VI and VII, splenocytes were stained with CD11b (M1/70, PE-Cy7), CD11c (N418, APC), CD43 (IBII, Alexa 488), Ly6C (AL-21, Pacific Blue), Ly6G (1A8, PE) and MHCII (25-9-17, Bio), using streptavidin (SA)-APC-Cy7 as a second stage reagent. Cells were sorted as described in Figure 4.3. L-DC were gated as $CD11b^{hi}CD11c^{lo}CD43^{+}Ly6C^{-}Ly6G^{-}MHCII^{-}$ cells. Resident monocytes (Resi mono) were gated as $CD11b^{hi}CD11c^{lo}CD43^{+}Ly6C^{+}Ly6G^{-}MHCII^{-}$ cells, while inflammatory monocytes (Infl mono) were gated as $CD11b^{hi}CD11c^{-}CD43^{+}Ly6C^{hi}Ly6G^{-}MHCII^{-}$ cells. In experiment VI, $CD43^{-}$ cDC were gated as described in Figure 4.3 as $CD11b^{+/-}CD11c^{hi}CD43^{-}MHCII^{+}Ly6C^{-}Ly6G^{-}$ cells. In experiment VII, neutrophils (Neu) were gated as described in Figure 4.3, $CD11b^{hi}CD11c^{-}CD43^{+}Ly6C^{+}Ly6G^{+}MHCII^{-}$ cells. Sorted APC were used to activate OT-I cells as described in Figure 5.3. OT-I T cells were sorted from OT-I mice as $PI^{-}Thy1.2^{+}V\alpha2^{+}CD4^{-}$ cells. Cells were cocultured with APC in T cell:APC ratios of 33:1, 100:1, 300:1 and 900:1. After 72 hours, $CD8^{+}$ OT-I T cells were gated as $PI^{-}CD11b^{-}Thy1.2^{+}V\alpha2^{+}$ cells, and assessed flow cytometrically for CFSE dilution as an indicator of proliferation. OT-I T cells alone served as a control (Con). Graphs show % proliferation of OT-I cells.

Expt VI




Expt VII




T cell : APC ratio

**Table 5.2 Ability of dendritic and myeloid subsets to activate $CD8^+$ T cells.**

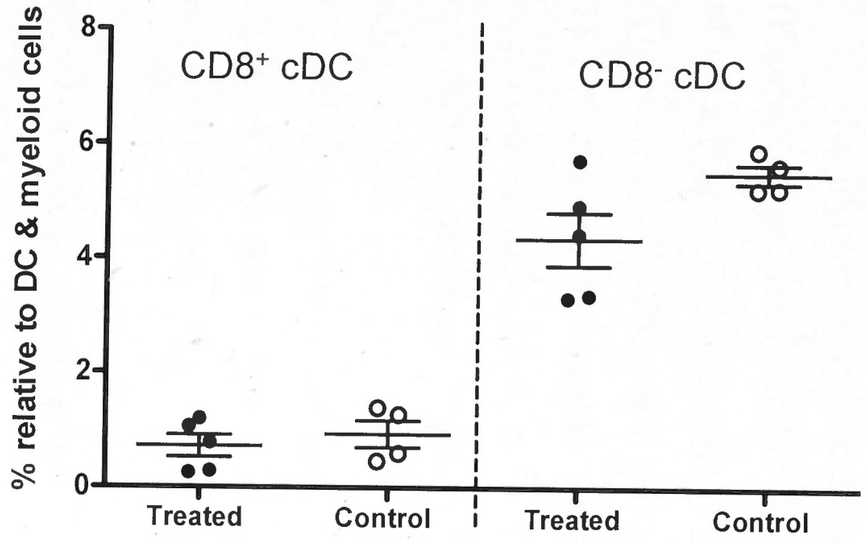
| Expt | Condition | T cell/APC ratio giving 50% maximum proliferation of OT-I T cells | | | | |
|---|---|---|---|---|---|---|
| | | L-DC | Resi mono[a] | Infl mono[b] | Neu[c] | $CD43^-$ cDC |
| VI | + LPS | 423 | 100 | < 33 | - | 300 |
| | - LPS | 300 | 100 | < 33 | - | 300 |
| VII | - LPS | 300 | 33 | 0 | << 33 | - |
| VIII | +LPS | 300 | 0 | - | << 33 | - |
| | - LPS | 141 | 0 | - | << 33 | - |

[a] Resi mono : Resident monocytes
[b] Infl mono : Inflammatory monocytes
[c] Neu : Neutrophils

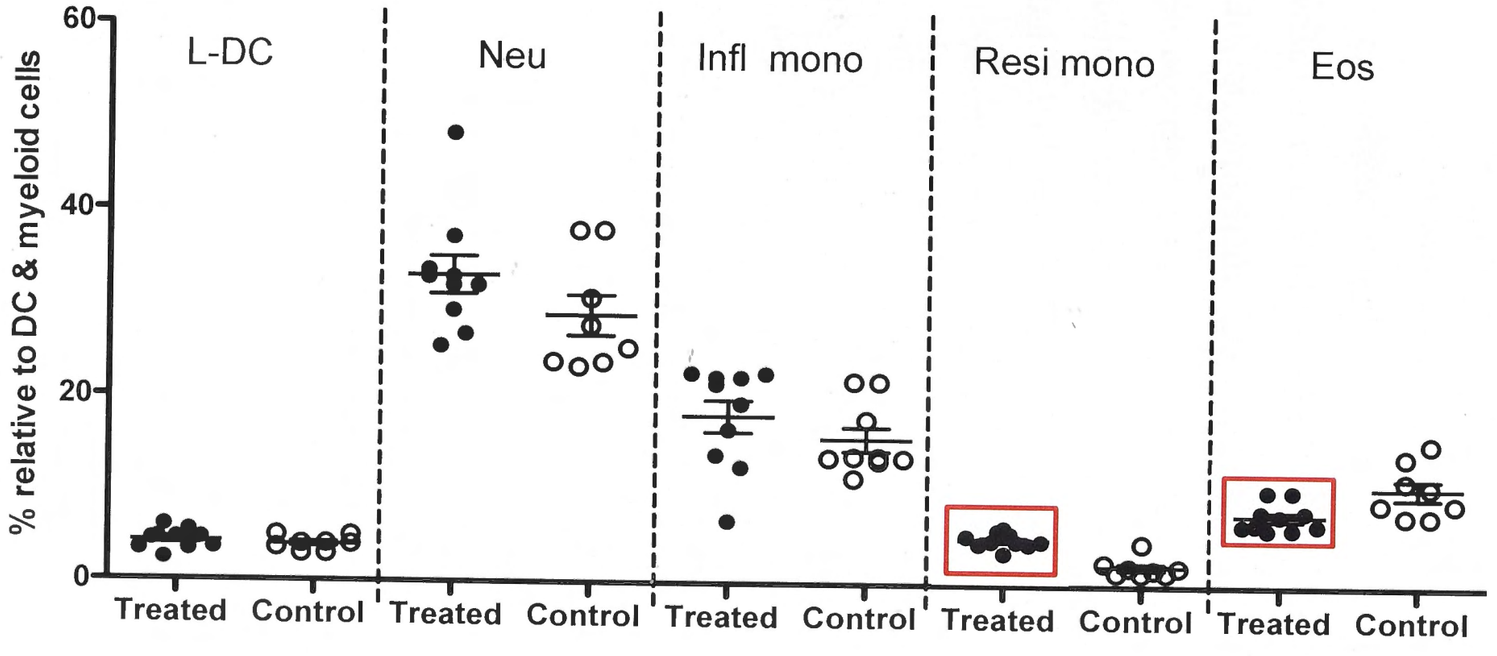
**Figure 5.5 Effect of *in vivo* cytochrome c treatment on myeloid and DC subset representation.**

The *in vivo* killing effect of cytochrome c was investigated in C57BL/6J mice. Cytochrome c (5mg/mouse) was delivered intravenously 6 hours prior to euthanasia for spleen collection. Control mice were given PBS. Spleen cells were prepared and stained for flow cytometry as described in the legend to Figure 5.3, and gated as described in Figure 5.2. Gates were set based on fluorescence minus one controls. Prior to flow cytometry, cells were stained with propidium iodide (PI, 1μg/ml) to delineate live ($PI^-$) cells. Individual mice were analysed. A bar is used to show mean values. A) Percent cDC relative to total dendritic and myeloid cell population. B) Percent myeloid subsets and L-DC relative to total dendritic and myeloid cell population. Red boxes indicate a significant change relative to wild type using Student's *t*-test (p value $\leq 0.01$).

A


% relative to DC & myeloid cells
8
6
4
2
0
CD8+ cDC
CD8- cDC
Treated
Control
Treated
Control

B


% relative to DC & myeloid cells
60
40
20
0
L-DC
Neu
Infl mono
Resi mono
Eos
Treated
Control
Treated
Control
Treated
Control
Treated
Control
Treated
Control

assessment of T cell proliferation. Consistent with the literature, a drop in the cross-presenting ability of $CD8^+$ cDC was observed when cells were treated with cytochrome c across multiple experiments (Figure 5.6). This effect was reflected by a weaker T cell proliferative response. Similarly, a drop in ability to cross-present was observed for $CD8^-$ cDC. Cytochrome c did reduce L-DC ability to cross-present antigen to $CD8^+$ T cells in one experiment, but this was not a consistent result and gave a 3-fold reduction in only one experiment (Figure 5.6 and Table 5.3). Cytochrome c treatment resulted in a consistent three-fold increase in the number of APC needed to give 50% maximum proliferation for both $CD8^-$ cDC and $CD8^+$ cDC (Table 5.3). While the cross-presentation capacity of $CD8^+$ cDC and $CD8^-$ cDC is clearly affected by cytochrome c treatment, the case for L-DC is less certain, with variable, small effects. One interpretation is that the cytosolic pathway is not used exclusively by the L-DC subset for cross-presentation of antigen.

Resident monocytes demonstrated weak ability to cross-prime $CD8^+$ T cells (Figure 5.4), and their numbers *in vivo* were not reduced by cytochrome c treatment of mice (Figure 5.5). Neutrophils showed little ability to cross-prime $CD8^+$ T cells (Figures 5.3 and 5.7), and required more APC and a T cell/APC ratio of less than 33:1 to generate measurable T cell proliferation (Table 5.4). Similarly, the T cell activating capacity of both resident and inflammatory monocytes was unaffected by cytochrome c treatment (Figure 5.7), and also required more APC and a T cell/APC ratio of less than 33:1 to induce measurable T cell proliferation (Table 5.4). Consistent with data shown in Table 5.3, cytochrome c treated L-DC in experiments VI and VII (Table 5.4) showed a minor and variable decrease in ability to cross-prime $CD8^+$ T cells following cytochrome c treatment.

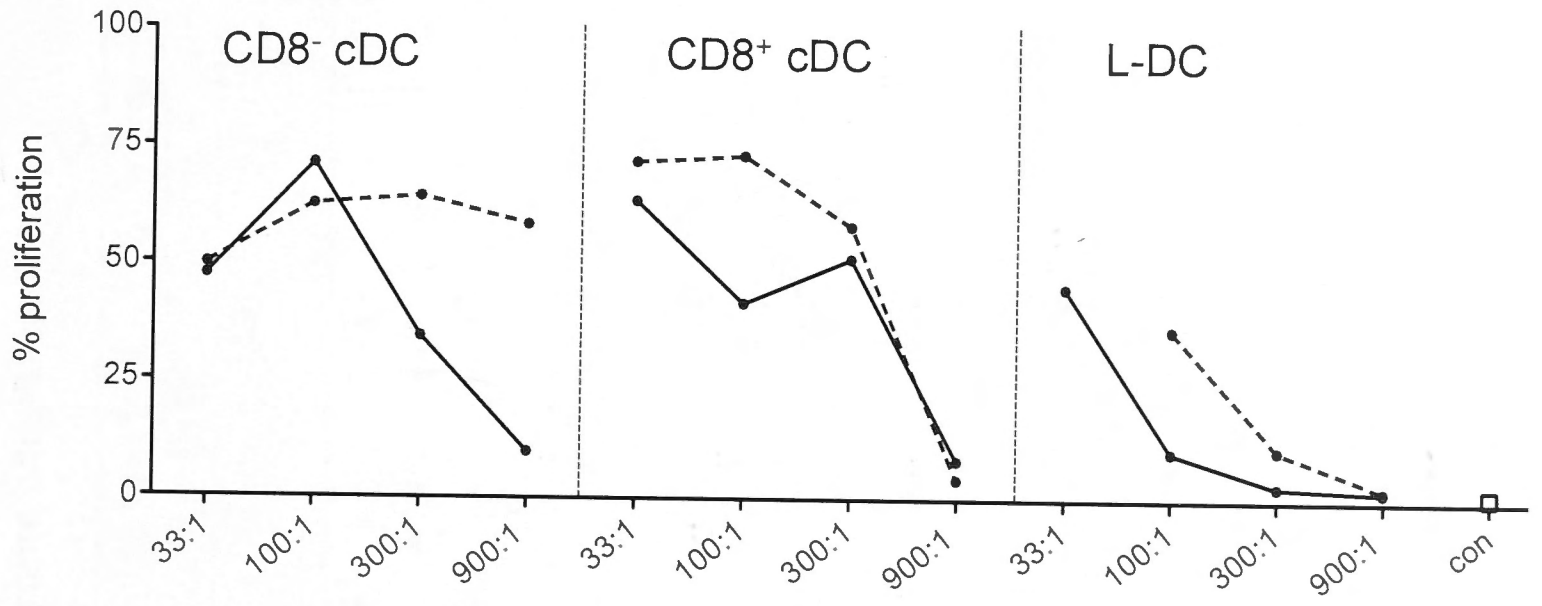
### 5.2.4 Ability of splenic dendritic and myeloid cells to activate $CD4^+$ T cells

Antigen presenting cells like DC and monocytes can take up exogenous antigen for processing and presentation on MHCII leading to activation of $CD4^+$ T cells. $CD8^-$ cDC have been described as the main APC for induction of $CD4^+$ T cell activation (Kronin et al., 2001). In contrast, LTC-DC have been described as $MHCII^-$ cells which lack ability to present antigen on MHCII for $CD4^+$ T cell activation (Tan et al., 2011). Lack of MHCII expression and inability to activate $CD4^+$ T cells can

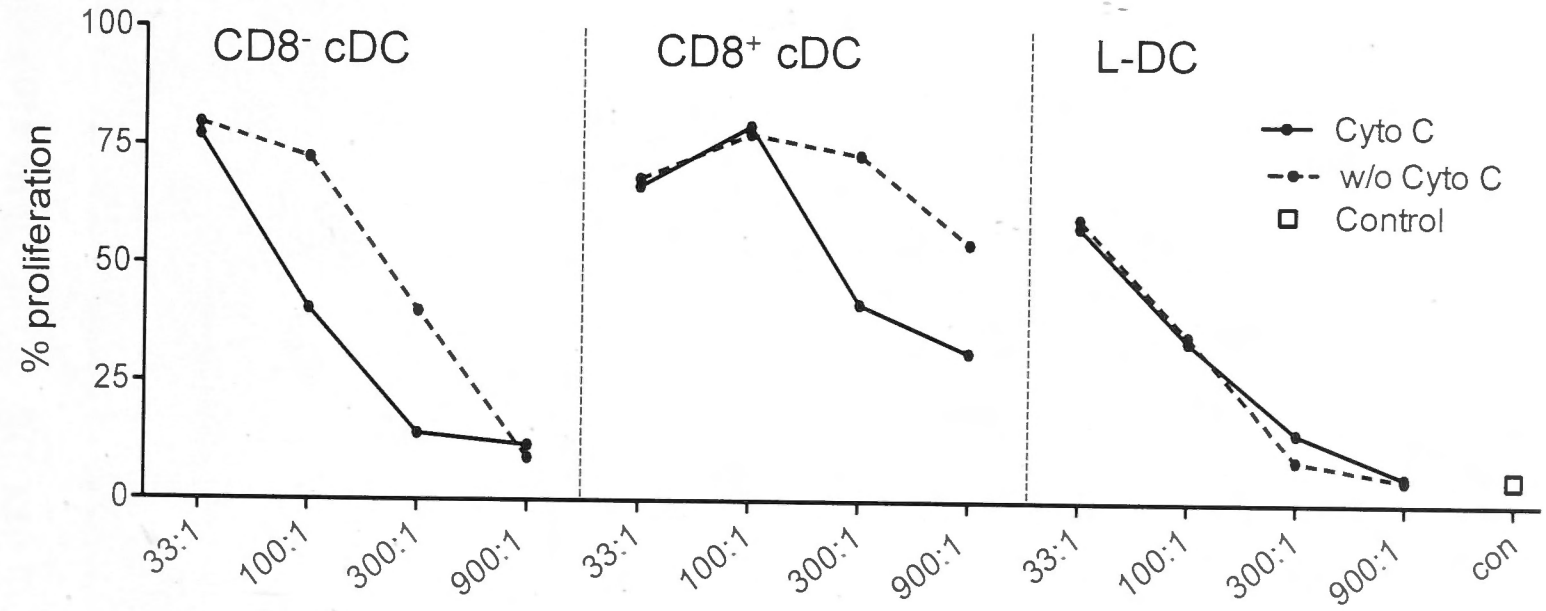
**Figure 5.6 Effect of cytochrome c on the cross-priming potential of isolated DC subsets.**

Splenocytes were harvested from Act-mOVA mice and prepared by red blood cells lysis and T/B cell depletion. In the two experiments shown, enriched splenocytes were stained as described in the legend to Figure 5.3. Cells were sorted as described in Figure 4.3. In both experiments, L-DC were gated as $CD11b^{hi}CD11c^{lo}Ly6C^{-}Ly6G^{-}MHCII^{-}$ cells. While $CD8^{+}$ cDC were gated as $CD11b^{-}CD11c^{hi}MHCII^{+}Ly6C^{-}Ly6G^{-}$ cells and $CD8^{-}$ cDC as $CD11b^{+}CD11c^{hi}MHCII^{+}Ly6C^{-}Ly6G^{-}$ cells. Diluting numbers of APC were plated followed by treatment with or without cytochrome c (6 mg/ml) for 2 hours prior to the addition of $10^{5}$ CFSE-labelled OT-I (TCR-tg) $CD8^{+}$ T cells, purified from OT-I mouse spleen through depletion of B cells, $CD4^{+}$ T cells, DC and myeloid cells using magnetic bead protocols. Cells were cocultured with APC in T cell:APC ratios of 33:1, 100:1, 300:1 and 900:1. After 72 hours, $CD8^{+}$ OT-I T cells were gated as $PI^{-}$ $CD11b^{-}Thy1.2^{+}V\alpha2^{+}$ cells, and assessed flow cytometrically for CFSE dilution as an indicator of proliferation. OT-I T cells alone served as controls (con). Graphs show % proliferation of OT-I cells.

Expt II




Expt III

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T cell : APC ratio

**Table 5.3 Activation of $CD8^+$ T cells following cytochrome c treatment.**

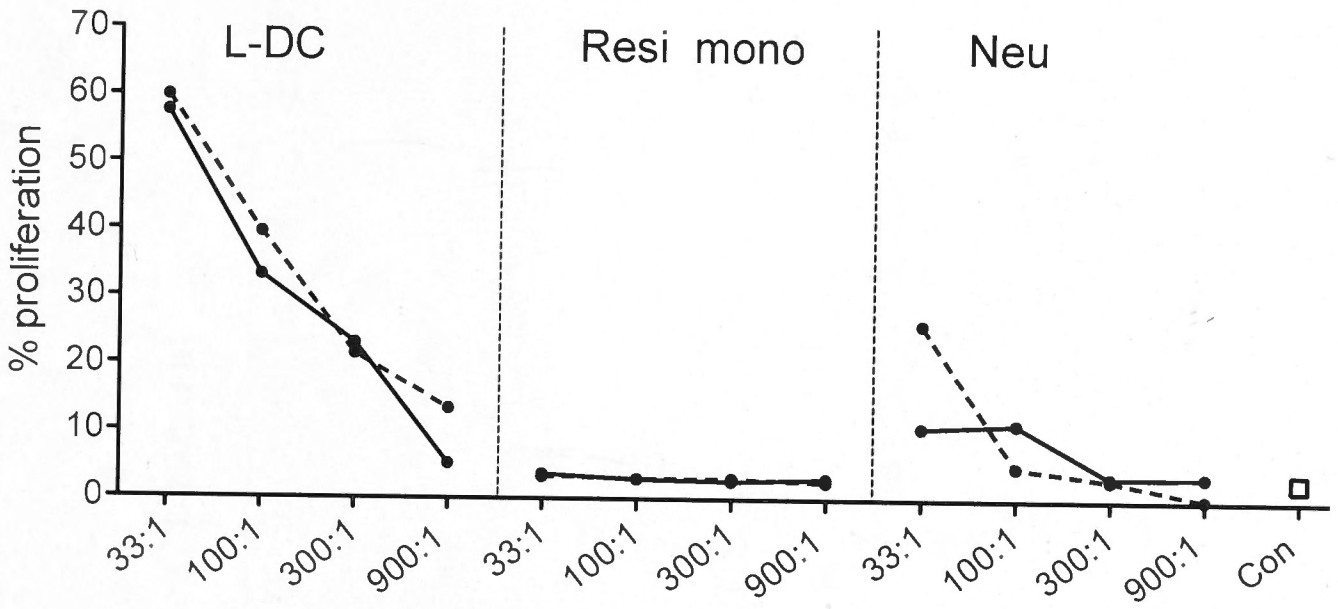
| Expt | Condition | T cell/APC ratio giving 50% maximum proliferation of OT-I T cells | | | |
|---|---|---|---|---|---|
| | | $CD8^-$ cDC | $CD8^+$ cDC | L-DC | Neu[a] |
| I | + Cyto C | 300 | 141 | 33 | << 33 |
| | - Cyto C | 900 | 300 | < 33 | << 33 |
| II | + Cyto C | 300 | 423 | 33 | - |
| | - Cyto C | >> 900 | 423 | 100 | - |
| III | + Cyto C | 300 | 100 | 100 | - |
| | - Cyto C | >> 900 | 300 | 141 | - |
| IV | + Cyto C | 423 | 100 | 100 | < 33 |
| | - Cyto C | 300 | 300 | 141 | < 33 |

[a] Neu : Neutrophils

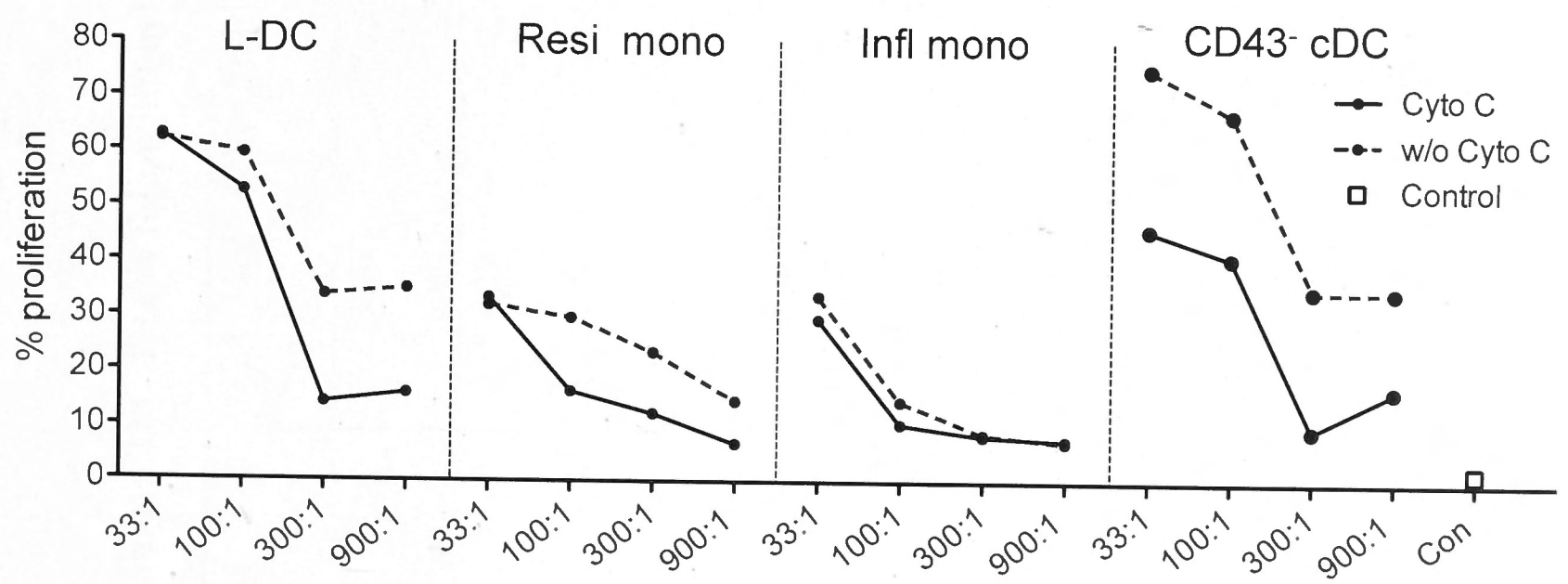
**Figure 5.7 Effects of cytochrome c on L-DC and myeloid cell subsets.**

Splenocytes were harvested from Act-mOVA mice and prepared by red blood cell lysis and T/B cell depletion. Enriched splenocytes were stained with CD11b (M1/70, PE-Cy7), CD11c (N418, APC), CD43 (IBII, Alexa 488), Ly6C (AL-21, Pacific Blue), Ly6G (1A8, PE) and MHCII (25-9-17, Bio), using streptavidin (SA)-APC-Cy7 as a second stage reagent. Cells were sorted as described in Figure 4.3. L-DC were gated as $CD11b^{hi}CD11c^{lo}CD43^{+}Ly6C^{-}Ly6G^{-}MHCII^{-}$ cells, while neutrophils (Neu) were gated as $CD11b^{hi}CD11c^{-}CD43^{+}Ly6C^{+}Ly6G^{+}MHCII^{-}$ cells. Resident monocytes (Resi mono) were gated as $CD11b^{hi}CD11c^{lo}CD43^{+}Ly6C^{+}Ly6G^{-}MHCII^{-}$ cells, while inflammatory monocytes (Infl mono) were gated as $CD11b^{hi}CD11c^{-}CD43^{+}Ly6C^{hi}Ly6G^{-}MHCII^{-}$ cells. In addition, cDC were gated as $CD11b^{+/-}CD11c^{hi}CD43^{-}MHCII^{+}Ly6C^{-}Ly6G^{-}$ cells. Cross-presenting capacity of subsets in the presence and absence of cytochrome c treatment was assessed by ability to induce proliferation in OT-I $CD8^{+}$ T cells as described in Figure 5.6. OT-I T cells served as controls (Con). Graphs show % proliferation of OT-I cells.

Expt V




Expt VI




T cell : APC ratio

**Table 5.4 Activation of $CD8^+$ T cells following cytochrome C treatment.**

| Expt | Condition | T cell/APC ratio giving 50% maximum proliferation of OT-I T cells | | | | |
|---|---|---|---|---|---|---|
| | | L-DC | Resi mono[a] | Infl mono[b] | Neu[c] | $CD43^+$ cDC |
| V | + Cyto C | 100 | << 33 | - | < 33 | - |
| | - Cyto C | 100 | << 33 | - | < 33 | - |
| VI | + Cyto C | 141 | < 33 | < 33 | - | 100 |
| | - Cyto C | 300 | < 33 | < 33 | - | 300 |

[a] Resi mono : Resident monocytes
[b] Infl mono : Inflammatory monocytes
[c] Neu : Neutrophils

therefore be used as another criterion for identification of an *in vivo* equivalent of LTC-DC. APC subsets were sorted from the spleens of Actm-OVA mice and compared for capacity to induce proliferation in $CD4^+$ T cells isolated from OT-II TCR-tg (anti $IA^b/OVA_{323-339}$) mice.

Consistent with the literature, both $CD8^+$ cDC and $CD8^-$ cDC were shown to present antigen for activation of $CD4^+$ T cells, with $CD8^-$ cDC as the stronger inducer (Pooley et al., 2001) (Figure 5.8). LPS had little effect as an activator of APC. L-DC induced no response in $CD4^+$ T cells even in the presence of LPS (Figure 5.8). Resident monocytes did activate a small proportion of $CD4^+$ T cells (Figure 5.8), while eosinophils, neutrophils and inflammatory monocytes completely lacked ability to present antigen for activation of $CD4^+$ T cells (Figure 5.8).

### 5.2.5 Induction of effector T cells by L-DC

The ability of APC to activate and induce proliferation in $CD8^+$ T cells does not necessarily translate to their ability to induce effector function. Experiments were therefore performed to compare the ability of L-DC, resident monocytes and $CD8^+$ cDC to induce cytotoxic effector function in $CD8^+$ T cells. An *in vivo* cytotoxic killing assay developed by Dr. Ben Quah (JCSMR, Canberra, ACT, Australia) was utilized here (Quah et al., 2012). Sorted $CD8^+$ OT-I (TCR-tg) T cells were delivered intravenously into mice one hour ahead of the delivery of APC sorted from Actm-OVA mice (Figure 5.9 A). Three doses of sorted APC were given to mice: 90,000, 9,000 or 900 cells. Six days later, labelled peptide-pulsed splenocytes were delivered intravenously to these mice to act as target cells for cytotoxic effectors primed by sorted APC (Figure 5.9 A). At one day after delivery of target cells, host splenocytes were harvested to quantitate % lysis of target cells (Figure 5.9 A). Ahead of adoptive transfer, target splenocytes were labelled with three different dyes used at four different concentrations. These were then pulsed with four different OVA peptides, either SIIN, N6, G4 or E1 used at six different concentrations, so creating a multiplex assay. The peptides used are discussed in the legend to Figure 5.9. OT-I T cells can recognise the SIIN peptide but not the G4 and E1 peptides included as negative controls. The N6 peptide is a variant of SIIN peptide with phenylalanine and glutamic acid removed, which is also recognised by OT-I T cells.

**Figure 5.8 Activation of $CD4^+$ T cells by different splenic APC.**

Splenocytes were harvested from Act-mOVA mice and prepared by red blood cell lysis and T/B cell depletion. In experiment I, T/B depleted splenocytes were stained as described in Figure 5.3. Cells were sorted as described in Figure 3.2, such that cDC were gated as $CD11c^{hi}MHC\text{-}II^+Ly6C^-Ly6G^-$ cells and further delineated to give $CD8^+$cDC and $CD8^-$cDC on the basis of CD11b expression. L-DC were gated as $CD11b^{hi}CD11c^{lo}Ly6C^-Ly6G^-MHC\text{-}II^-$ cells, while resident monocytes (Resi mono) were gated as $CD11b^{hi}CD11c^{lo}Ly6C^+Ly6G^-MHC\text{-}II^-$ cells. In experiments II and III, splenocytes were stained with an additional antibody specific for Siglec-F. Dendritic and myeloid cell subsets were gated as described in Figure 4.3. Both $CD8^-$cDC and L-DC were gated as described in experiment I but also as Siglec-$F^-$ cells. Eosinophils (Eos) were gated as $CD11b^{hi}CD11c^-Ly6C^+Ly6G^-Siglec\text{-}F^+$ cells, while neutrophils (Neu) were gated as $CD11b^{hi}CD11c^-Ly6C^+Ly6G^+Siglec\text{-}F^-$ cells. Resident monocytes were gated as $CD11b^{hi}CD11c^{lo}Ly6C^+Ly6G^-Siglec\text{-}F^-$ cells, while inflammatory monocytes (Infl mono) were gated as $CD11b^{hi}CD11c^-Ly6C^{hi}Ly6G^+Siglec\text{-}F^-$ cells. Diluting numbers of APC were plated, followed by treatment with or without lipopolysaccharide (LPS:10ug/ml) for 2 hours, prior to the addition of $10^5$ CFSE-labelled OT-II (TCR-tg) $CD4^+$ T cells, sorted as $PI^-Thy1.2^+V\alpha2^+CD8^-$ cells. Cells were cocultured in T cell:APC ratios of 33:1, 100:1, 300:1 and 900:1. After 72 hours, $CD4^+$ OT-II T cells were gated as $PI^-CD11b^-Thy1.2^+V\alpha2^+$ cells, and assessed flow cytometrically for CFSE dilution as an indicator of proliferation. OT-II T cells alone served as controls (Con). Graphs show % proliferation of OT-II cells.

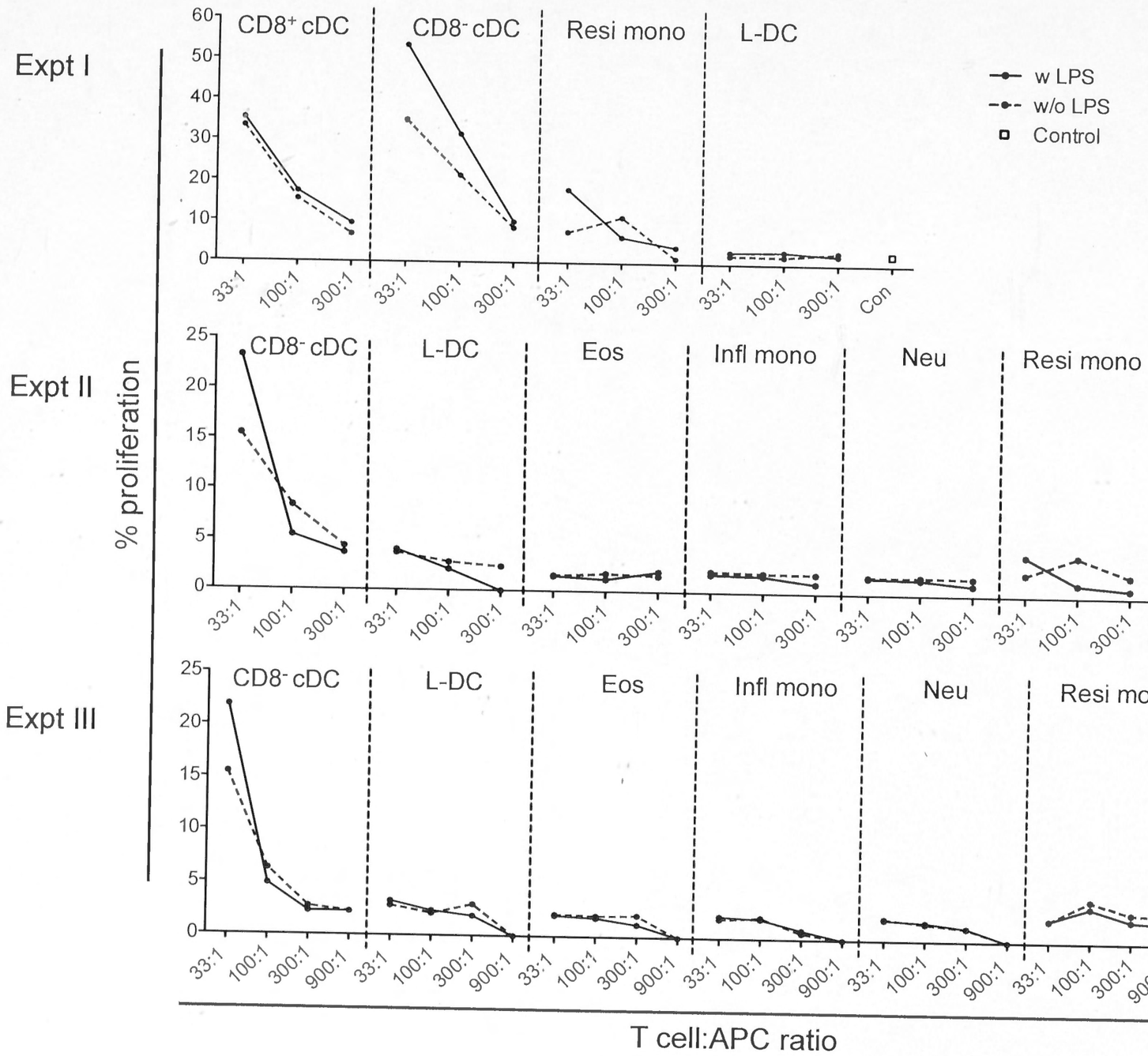
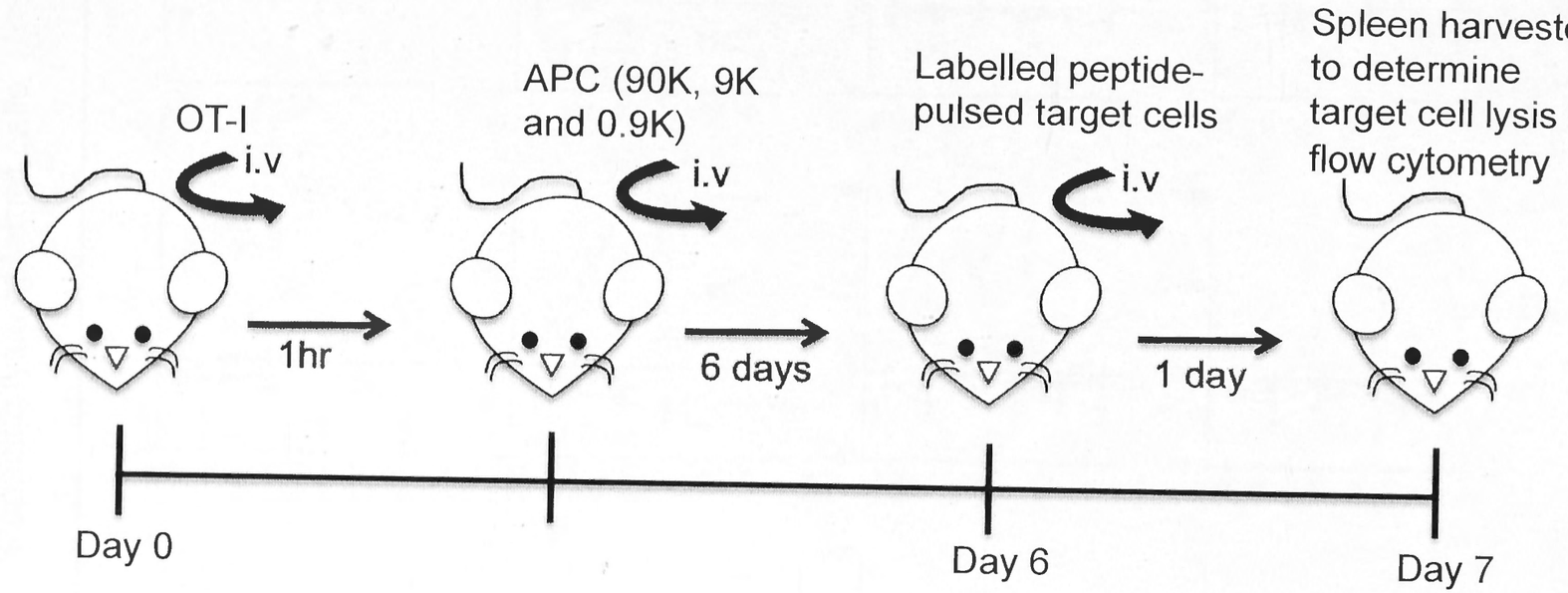

Expt I
Expt II
Expt III
% proliferation
T cell:APC ratio
CD8+ cDC
CD8- cDC
Resi mono
L-DC
Eos
Infl mono
Neu
w LPS
w/o LPS
Control
33:1
100:1
300:1
900:1
Con

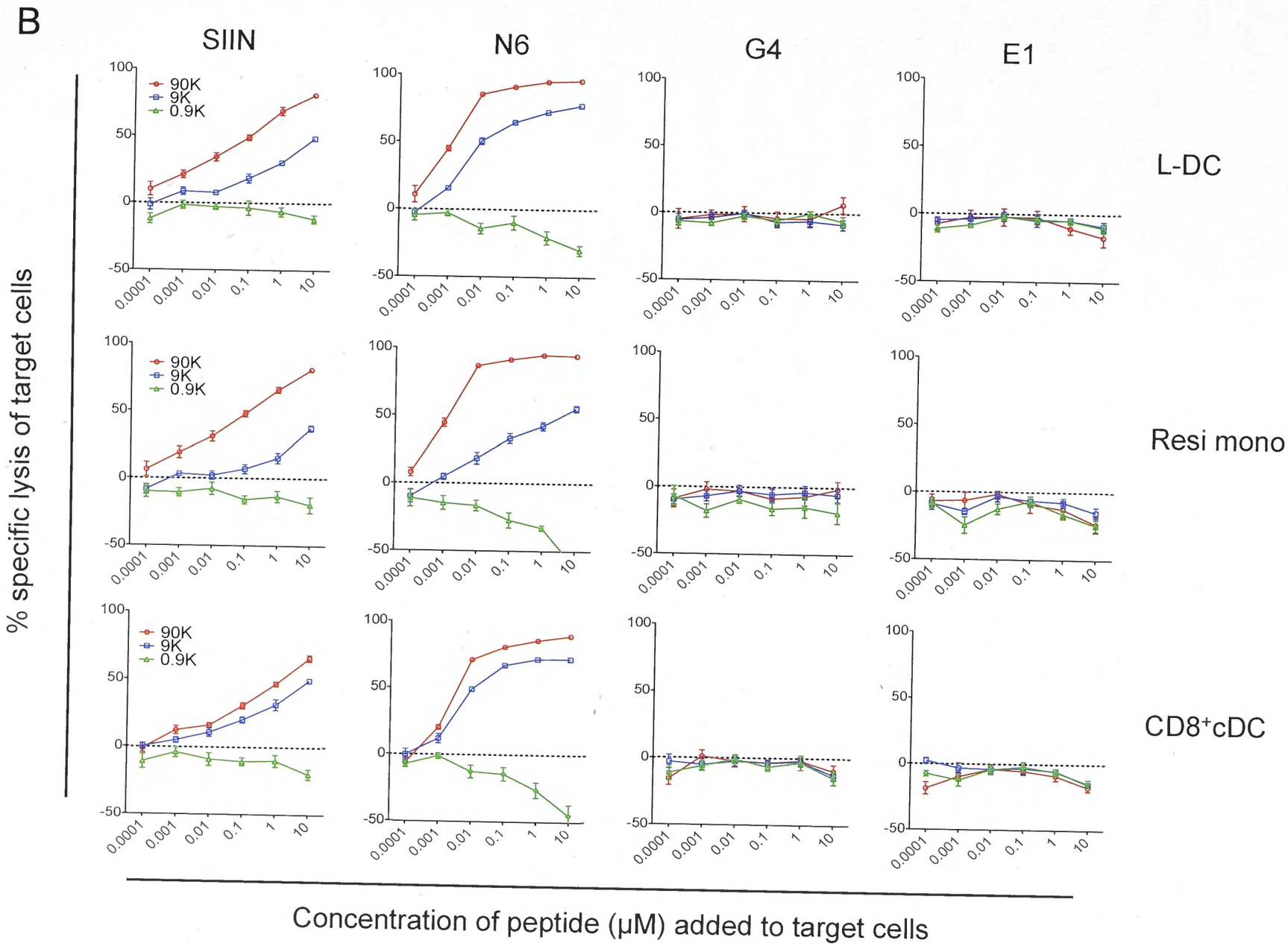
**Figure 5.9 Ability of splenic APC subset to induce cytotoxic T lymphocytes.**

The ability of APC subsets to induce cytotoxic effector function in $CD8^+$ T cells was assessed by measuring lysis of OVA peptide-pulsed target cells in a fluorescent target assay (FTA). A) The experimental procedure is shown as a timeline. On Day 0, $CD8^+$ T cells from OT-I TCR-Tg mice were prepared by RBC lysis of splenocytes and sorting for $PI^-Thy1.2^+V\alpha2^+CD4^-$ cells. OT-I T cells ($3.5 \times 10^6$) were delivered intravenously (i.v.) into host mice (C57BL/6). An hour later, several APC subsets sorted from Actm-OVA mice were also delivered into host mice. These were sorted as described in Figure 4.3, and three cell doses (90K, 9K or 0.9K) were given i.v.. In order to measure the effector function of activated $CD8^+$ T cells after 7 days, B6.SJL splenocytes as targets were prepared and adoptively transferred i.v. on Day 6. Target cells were labelled with several concentrations of 3 different dyes for later identification as described in Section 2.5.3. Overall, labelled target cells were then pulsed with 6 different concentrations of 4 distinct OVA peptides: SIINFEKL (SIIN), GLEQLESIINFEKL (N6), SIIGFEKL (G4) and EIINFEKL (E1). Specific killing of the distinctly labelled, antigen-pulsed target cells was determined by flow cytometric analysis to determine the number of target cells remaining in the test mouse compared with the control mouse given OT-I T cells only. Calculation of target lysis involved the formula described in Section 2.5.3. B) % specific lysis of target cells pulsed with different concentrations of peptides by OT-I T cells primed with three different APC types. Data is expressed as mean ± SE (n= 6).

A


OT-I
i.v
1hr
APC (90K, 9K and 0.9K)
i.v
6 days
Labelled peptide-pulsed target cells
i.v
1 day
Spleen harvested to determine target cell lysis by flow cytometry
Day 0
Day 6
Day 7

B


SIIN
N6
G4
E1
L-DC
Resi mono
CD8+cDC
90K
9K
0.9K
100
50
0
-50
0.0001
0.001
0.01
0.1
1
10
% specific lysis of target cells
Concentration of peptide (μM) added to target cells

The APC subsets of L-DC, resident monocytes and $CD8^+$ cDC each induced CTL which lysed target cells pulsed with SIIN and N6 peptides, but not target cells pulsed with the G4 or E1 peptides (Figure 5.9 B). In addition, the level of lysis of target cells reduced with decreasing concentration of peptides used to pulse APC, and the numbers of APC used to prime host mice (Figure 5.9 B). In order to directly compare APC subsets, the concentration of peptide required to prime CTL for 50% lysis of target cells was determined for each APC. With N6 peptide, CTL generated by priming with 9,000 resident monocytes required 100 times more peptide to induce 50% lysis of target cells, when compared with L-DC and $CD8^+$ cDC. After priming with 90,000 APC, cytotoxic T lymphocytes generated with $CD8^+$ cDC as APC, required 10 times more SIIN peptide to give 50% lysis of target cells by comparison with L-DC and resident monocytes (Table 5.5). When the number of priming APC was reduced to 9,000, CTL generated by resident monocytes required a higher concentration of SIIN to induce 50% lysis of target cells when compared with L-DC and $CD8^+$ cDC (Table 5.5). Overall, resident monocytes induced a weaker CTL response compared with L-DC and $CD8^+$ cDC. The latter two APC appear to induce similar cytotoxic effector responses.

**Table 5.5 Capacity of splenic subsets to induce cytotoxic T cells.**

| Peptide | No. APC from Actm-OVA mice used for priming * + Targets: | Concentration of peptide (uM) required to activate CTL for 50% lysis of targets | | |
|---|---|---|---|---|
| | | L-DC | Resi Mono | $CD8^+$cDC |
| SIIN | 90 K | 0.1 | 0.1 | 1.0 |
| | 9 K | 10.0 | >>10 | 10.0 |
| | 0.9 K | 0 | 0 | 0 |
| N6 | 90 K | 0.001 | 0.001 | 0.001 |
| | 9 K | 0.01 | 1.0 | 0.01 |
| | 0.9 K | 0 | 0 | 0 |
| G4 | 90 K | 0 | 0 | 0 |
| | 9 K | 0 | 0 | 0 |
| | 0.9 K | 0 | 0 | 0 |
| E1 | 90 K | 0 | 0 | 0 |
| | 9 K | 0 | 0 | 0 |
| | 0.9 K | 0 | 0 | 0 |

* Mice were also given sorted OT-I $CD8^+$ T cells ($3.5 \times 10^6$) prior to APC.
+ APC were sorted from spleens of mACT-OVA mice. These included L-DC, resident monocytes (Resi mono) and $CD8^+$ cDC.

## 5.3 Discussion

There have been many reports suggesting that the route of antigen uptake can determine the processing and presentation pathway used for T cell activation (Burgdorf et al., 2007; Burgdorf et al., 2006; Burgdorf et al., 2008). Burgdorf et al. (2006, 2007, 2008) demonstrated that soluble antigen uptake in DC via pinocytosis can lead to strikingly different outcomes in terms of activation of T cells compared with mannose receptor-mediated uptake. They showed that uptake of antigen via the mannose receptor led exclusively to $CD8^+$ T cell activation as evidence of cross-presentation, while uptake via pinocytosis led to the activation of $CD4^+$ T cells. However, their study did not distinguish the capacity of cDC, since they isolated $CD11c^+$ splenocytes via density centrifugation and microbead technology rather than by sorting, and compared only the cross-priming ability of the heterogeneous population of $CD11c^+$ splenocytes, bone marrow-derived DC and bone marrow-derived macrophages. The relationship between antigen uptake capacity and cross-presentation capacity has been studied here using carefully sorted myeloid and dendritic subsets characterised in spleen.

Here it is shown that splenic DC and myeloid subsets not only differ in phenotype, but also have distinct functional capacity. Aspects of immune function studied in this chapter help to distinguish these splenic subsets further. $CD8^+$ cDC and $CD8^-$ cDC can be distinguished from myeloid subsets by their high endocytic ability, their strong cross-priming ability, and their ability to induce both CD4 and CD8 T effector functions. By comparison with myeloid subsets, cDC subsets were capable of endocytosing soluble antigen, but showed weaker capacity for mannose receptor-mediated endocytosis. Amongst the myeloid subsets, monocytes, eosinophils and neutrophils lacked ability to activate $CD4^+$ T cells, and only resident monocytes demonstrated some ability to cross-prime $CD8^+$ T cells. In terms of endocytic capacity, resident monocytes and inflammatory monocytes demonstrated superior ability to endocytose antigens via the mannose receptor, and resident monocytes showed the strongest ability to endocytose soluble antigen. The $Ly6C^-$ $Ly6G^-$ L-DC candidate identified in Chapter 4 demonstrated strong mannose receptor-mediated uptake of antigen but weak capacity to endocytose soluble antigen. This L-DC candidate also displayed stronger ability than myeloid subsets to

cross-prime $CD8^+$ T cells (Figure 5.3 and 5.4), and lacked ability to activate $CD4^+$ T cells consistent with former evidence from LTC-DC.

Previously, a functional mannose receptor was considered to be a marker of cross-presentation ability of splenic APC subsets (Burgdorf et al., 2006; Burgdorf et al., 2010). However, here we show that antigen uptake via mannose receptors is not related to cross-presentation capacity. Both resident and inflammatory monocytes demonstrate potent ability to take up mannan-FITC (Figure 5.2) but do not cross-prime $CD8^+$ T cells (Figure 5.2). At 3 hours post delivery of mannan, close to 90% of inflammatory monocytes and 75% of resident monocytes had taken up mannan-FITC (Figure 5.3 and 5.4). By comparison, only 52% of $CD8^+$ cDC, 48.5% of L-DC and 34% of $CD8^-$ cDC had taken up mannan (Figure 5.2). Inflammatory and resident monocytes did not cross-present antigen at the level seen for $CD8^+$ cDC, $CD8^-$ cDC and L-DC, although, L-DC and $CD8^+$ cDC showed similar uptake of mannan-FITC, but variable cross-presenting ability.

The hypothesis that antigen uptake via the mannose receptor is an indicator of cross-presentation ability is therefore disputed on the basis of data presented here. Previously, Segura et al. (2010) demonstrated that gene knock-out of aminopeptidase IRAP, encoding an enzyme essential for proteolytic breakdown of antigen in the endosome, did not affect the cross-presenting ability of $CD8^+$ cDC. Similarly, the knock down of mannose receptor in $CD8^+$ cDC did not impact on ability to cross-present antigen (Segura et al., 2009). However, gene knock down of IRAP and mannose receptor did adversely impact the ability of *ex vivo* isolated monocyte-derived DC (mo-DC) to cross-prime (Segura et al., 2009). One explanation for these discrepant findings could be that DC have multiple receptors on the cell membrane which participate in the endocytosis of antigen, also consistent with their ability to clear apoptotic cells from the environment (Savill et al., 2002). Thus, for $CD8^+$ cDC at least, receptors other than the mannose receptor must contribute to the uptake of antigens, and these must contribute to cross-priming of $CD8^+$ T cells in mannose receptor and IRAP knockout mice.

Early studies described the uptake of antigen via pinocytosis as an important element in the activation of $CD4^+$ T cells (Burgdorf et al., 2007). Both $CD8^+$ cDC

and $CD8^-$ cDC demonstrate potent ability to take up antigen via pinocytosis and are both strong activators of $CD4^+$ T cells (Figure 5.1 A) (Belz et al., 2005; Schnorrer et al., 2006). Resident monocytes were found to be the most pinocytic over a six hour time course (Figure 5.1 A), but these cells do not have the capacity to activate $CD4^+$ T cells. Such activation capacity is due to a lack of MHCII expression rather than pinocytosis ability. One distinguishing trait of L-DC is their inability to activate $CD4^+$ T cells. This is likely due to their absence of MHCII expression, rather than their weak pinocytic ability in comparison with monocytes (Figure 5.1 C).

Immature DC have been described as highly endocytic until activation and maturation, after which endocytic ability is reduced (Guermonprez et al., 2002; Steinman and Swanson, 1995; Wilson et al., 2004). This has been proposed as a possible mechanism for retaining antigen for cross-presentation (Savina and Amigorena, 2007). The *in vitro* studies on cross-presentation, involving pulsing APC with soluble OVA then measuring cross-presentation via proliferation of $CD8^+$ T cells, are not reflective of the *in vivo* environment which allows multiple cell interactions (Burgdorf et al., 2007; Burgdorf et al., 2006; Burgdorf et al., 2008; Segura et al., 2009). During the inflammatory state, antigen *in vivo* could be both cell-associated and soluble in blood. Different APC could be needed to clear dead cells or cell debris, than are needed to take up soluble antigen from blood. Since each of the monocyte, L-DC and cDC subsets described here is able to take up soluble antigen as a spleen subset, they may be well placed to clear blood-borne antigens in spleen.

In order to gain an accurate picture of the ability of splenic subsets to cross-present cell-associated antigen, subsets were sorted from Actm-OVA mice and their cross-priming ability investigated *in vitro*. Actm-OVA mice display constitutive expression of membrane-bound OVA. Antigen presenting cells in Actm-OVA mice acquire OVA protein during clearance of dead cells and cell debris in tissues and blood. In terms of experiments described here, no pulsing of APC with OVA was required, and the Actm-OVA mouse system provided an excellent model for presentation of cell-associated antigen. One limitation of the model is that some level of OVA could be processed via the endogenous pathway and become loaded on to the MHCI molecule for presentation to $CD8^+$ T cells. Cells which do not cross-

present were therefore employed as negative controls. Neutrophils in the absence of LPS stimulation showed no ability to cross-prime $CD8^+$ T cells giving values close to zero and to T cell only controls (Figure 5.3), consistent with other reports in the literature (Savina and Amigorena, 2007). The amount of OVA being processed via the endogenous pathway is negligible in neutrophils, although LPS treated neutrophils showed very weak ability to cross-prime $CD8^+$ T cells (Figure 5.3). However, bone marrow and peritoneal neutrophils can cross-prime $CD8^+$ T cells under inflammatory although not steady-state conditions (Beauvillain et al., 2007). In experiments described here, neutrophils without LPS stimulation were therefore used as control APC.

In line with the literature, both $CD8^+$ cDC and $CD8^-$ cDC showed superior ability to cross-prime $CD8^+$ T cells in comparison with neutrophils (Figure 5.3) (Heath et al., 2004), while L-DC showed slightly weaker capacity (Figure 5.3). Under protocols used here, LPS stimulation had no or little impact on L-DC cross-priming ability, while both subsets of cDC induced a better response under LPS stimulation (Figure 5.3). Gene profiling in Chapters 3 and 6 revealed that L-DC express both *Tlr2* and *Tlr4*, which encode TLR essential for signalling upon LPS activation. Gene expression levels of *Tlr2* and *Tlr4* were comparable with the cDC (Good et al., 2012; McCurdy et al., 2001). In addition, L-DC also express *Cd14* which encodes a co-receptor for TLR4 at levels comparable with cDC subsets. Thus, the lack of response towards LPS in L-DC is not due to the absence of receptors for LPS, but rather insensitivity towards LPS activation. Another possible explanation could be a lack of co-receptors of TLR, such as monomeric recombinant (MD-2) and/or LPS-binding protein in L-DC.

A comparison of the ability of different APC subsets to cross-prime revealed that $CD8^-$ cDC were consistently better than $CD8^+$ cDC. Cross-priming ability had been previously described in $CD8^+$ cDC, while $CD8^-$ cDC were found to be much weaker in those experiments (Den Haan et al., 2000; Heath et al., 2004; Rock et al., 1990). Those studies however used cultured cDC, involved *ex vivo* pulsing of antigen, or were performed in disease models (Belz et al., 2005; Schnorrer et al., 2006). Studies here showed that $CD8^-$ cDC can endocytose and retain antigen, such that antigen capture is not a limiting factor in the ability of $CD8^-$ cDC to cross-prime

$CD8^+$ T cells. Since $CD8^-$ cDC have been described as better inducers of $CD4^+$ T cell activation (Dudziak et al., 2007; Maldonado-López et al., 1999; Villadangos and Schnorrer, 2007), one hypothesis is that in normal mice, $CD8^-$ cDC have the machinery for cross-presentation, but may preferentially use the MHCII antigen processing pathway for $CD4^+$ T cell activation. $CD8^-$ cDC sorted from the Actm-OVA mice would have been exposed to cell-associated OVA constantly which may predispose cells to cross-presentation. The cross-priming ability of APC in the Actm-OVA animal model may therefore not be reflective of normal animals.

Cross-priming of $CD8^+$ T cells is considered an exclusive property of DC (Belz et al., 2005; Den Haan et al., 2000; Schnorrer et al., 2006; Shortman and Heath, 2010). However, there have been reports that macrophages can also cross-present antigen (Harding and Song, 1994; Kovacsovics-Bankowski et al., 1993; Norbury et al., 1995; Pfeifer et al., 1993). Resident monocytes, which are considered to be the precursors of macrophages, were investigated here for their ability to cross-prime $CD8^+$ T cells since there are no reports of the cross-presentation capacity of monocytes. Resident monocytes did demonstrate ability to cross-prime $CD8^+$ T cells in two out of four experiments. However, their ability was very weak in comparison with cDC and L-DC. The addition of LPS did improve ability to cross-prime, suggesting that some resident monocytes may have become activated upon LPS stimulation, and may have even differentiated to give macrophages.

Cytochrome c treatment can be used effectively to induce apoptosis specifically in cells with cross-presenting ability (Ming et al., 2008). Cytochrome c is endocytosed by APC in the same way as antigen, and released into the cytoplasm for cross-presentation via the cytoplasmic pathway. When cytochrome c enters the cytoplasm after uptake, it binds to apoptotic protease activating factor 1 (Apaf-1) to form an apoptosome which then induces a caspase cascade resulting in cell death (Jiang and Wang, 2004). Contrary to previous reports (Ming et al., 2008), we have shown here that cDC numbers in the animal are not reduced following intravenous delivery of cytochrome c (Figure 5.5). One problem could be the known variation amongst the batches of cytochrome c (personal communication with L.L.Ming, WEHI, Melbourne, Australia). However when cytochrome c was used *in vitro* to treat $CD8^+$ cDC and $CD8^-$ cDC, there was a 3 fold reduction in the capacity of cDC

to induce $CD8^+$ T cell proliferation. By comparison, cytochrome c treated L-DC showed only a 1.5 fold reduction in their ability to cross-prime $CD8^+$ T cells. *In vivo* treatment with cytochrome c might require much higher concentrations to induce the same level of cell death as achieved *in vitro*. The impact of cytochrome c treatment on cDC subsets *in vitro* suggests that the cytosolic pathway may be primarily employed for cross-presentation by these cells. Effect of cytochrome c on L-DC is variable, thus L-DC might use both the cytosolic and vacuolar pathways for cross presentation. Cytochrome c treatment did not affect the numbers of neutrophils and monocytes present when given *in vivo* (Figure 5.5). Furthermore, when myeloid subsets were isolated and tested in the cross-presentation assay after *in vitro* cytochrome c treatment, no inhibition of function was observed for neutrophils (Figure 5.6 and 5.7). Resident monocytes did not induce $CD8^+$ T cell proliferation in two out of three experiments performed.

Both cDC and L-DC subsets showed strong capacity for cross-priming leading to activation of $CD8^+$ T cells, which is essential in the generation of cytotoxic T lymphocytes (Barry and Bleackley, 2002). Cytotoxic T lymphocytes play a dual role in maintaining self-tolerance by lysing self targets, and by lysing infected cells or cancerous cells. The ability of cytotoxic T lymphocytes generated *in vitro* by antigen-pulsed L-DC, $CD8^+$ cDC and resident monocytes was investigated using a fluorescent target array to detect lysis *in vivo*. This was developed by Dr Ben Quah (Quah et al., 2012). Cytotoxic T lymphocytes generated by L-DC presentation of OVA, lysed target cells pulsed with the SIIN and N6 peptides of OVA, but not the G4 and E1 peptides. This demonstrated that lysis of target cells was antigen-specific, since OT-I $CD8^+$ T cells only recognise SIIN and N6 peptides in the context of MHCI. Similarly, both $CD8^+$ cDC and resident monocytes generated cytotoxic T lymphocytes which gave antigen-specific lysis of target cells. However, resident monocytes required a much higher concentration of peptide on target cells to give a similar level of lysis. Cytotoxic T lymphocytes generated by priming with 9000 resident monocytes required 100 times more N6 peptide on target cells than did L-DC or $CD8^+$ cDC to achieve the same level of target lysis (Table 5.5). Thus, resident monocytes are weaker inducers of cytotoxic T lymphocytes than L-DC and cDC, consistent with a lack of cross-presenting ability. In contrast, both $CD8^+$ cDC and L-DC demonstrate strong ability to generate a CTL response.

Activation of $CD4^+$ T cells is essential in adaptive immunity to activate B cells and macrophages. $CD8^-$ cDC have been described as strong inducers of $CD4^+$ T cell activation (Behrens et al., 2004). Consistent with the literature, $CD8^-$ cDC were found here to be the strongest inducers of $CD4^+$ T cell proliferation, followed by $CD8^+$ cDC (Figure 5.8). LTC-DC as $MHCII^-$ cells lacked ability to activate $CD4^+$ T cells (Tan et al., 2011). L-DC defined here as an *in vivo* $MHCII^-$ subset, did not induce proliferation of $CD4^+$ T cells, while resident monocytes induced low level proliferation (Figure 5.8), and the level of response was much lower than for cDC subsets. Lastly neutrophils, eosinophils and inflammatory monocytes, which lack MHCII expression, also failed to induce proliferation of $CD4^+$ T cells.

While both L-DC and resident monocytes share a similar phenotype (Chapter 4), they are shown here to be distinct in terms of function. Even though resident monocytes demonstrate strong ability to uptake and retain antigen, they lack ability to cross-prime $CD8^+$ T cells. The relationship between L-DC and resident monocytes will be explored further in Chapter 6 by gene profiling these subsets, along with other splenic myeloid and cDC subsets studied here.

Chapter 6

# Molecular definition of splenic dendritic and myeloid cell subsets

## 6.1 Introduction

In order to delineate the lineages of dendritic cells (DC) and tissue macrophages more completely, both Gautier et al. (2012) and Miller et al. (2012) employed transcriptome analysis to compare related subsets of cells isolated from different tissue sites. Gautier et al. (2012) identified and isolated many macrophage subsets from different tissues and compared their gene expression profiles to identify a core macrophage gene signature profile that could be used to delineate tissue macrophage subsets from DC. Similarly, Miller et al. (2012) identified various DC subsets in different tissue sites and compared their gene expression profiles to identify a core tissue DC profile that distinguishes cells of the dendritic lineage from tissue macrophages. In addition, Miller et al. (2012) also identified transcriptional regulators involved in the commitment of myeloid progenitor cells to the DC lineage.

Transcriptome analysis has been undertaken here to analyse the relationship between splenic myeloid subsets including the newly identified L-DC in relation to cDC, and also with the myeloid subsets of inflammatory monocytes, resident monocytes and eosinophils. In an earlier transcriptome analysis performed in Chapter 3, it was concluded that the L-DC subset defined as $CD11b^{hi}CD11c^{lo}MHCII^{-}CD8^{-}$ cells, was a heterogeneous population with gene expression indicative of myeloid lineage cells, but with cells having antigen presenting function akin to the dendritic lineage of antigen presenting cells (APC). In addition, this subset of 'candidate' L-DC also expressed genes expressing inhibitory function towards T cells. With the new antibody staining and delineation protocols established in Chapter 4, and with confirmation of subset identities made in Chapters 4 and 5 through both functional and morphological analysis, it is now possible to better define the L-DC, cDC and myeloid subsets.

Putative $CD11b^{hi}CD11c^{lo}MHCII^{-}CD8^{-}$ L-DC identified in Chapter 3 were further delineated to give 3 candidates on the basis of Ly6C and Ly6G expression as described in Chapter 4. Morphological and phenotypic studies in Chapter 4 further defined the candidate L-DC as a $CD11b^{hi}CD11c^{lo}Ly6C^{-}Ly6G^{-}CD43^{+}Siglec\text{-}F^{-}$ `subset. In addition, splenic resident monocytes and eosinophils were redefined as $CD11b^{hi}CD11c^{lo}Ly6C^{+}Ly6G^{-}CD43^{hi}Siglec\text{-}F^{-}$ and $CD11b^{hi}CD11c^{-}Ly6C^{+}Ly6G^{-}$

$CD43^{+}Siglec\text{-}F^{+}$ cells, respectively. The identity of the candidate L-DC subset was confirmed via functional assays performed in Chapter 5. Despite new understanding about these splenic dendritic and myeloid subsets, the lineage relationship between L-DC and other dendritic and myeloid cell subsets in spleen remains unknown. To further analyse their relationship in terms of lineage, further gene profiling analyses have been undertaken.

## 6.2 Results

### 6.2.1 Transcriptome analysis of splenic myeloid subsets

Subsets of $CD8^+$ cDC, $CD8^-$ cDC, resident monocytes, inflammatory monocytes, eosinophils and L-DC were sorted from spleens of C57BL/6J mice according to the staining procedure used for subset identification in Figure 4.3, and sorted according to the strategy outlined in Figure 6.1.

High purity RNA was extracted from sorted L-DC, $CD8^+$ cDC, $CD8^-$ cDC, inflammatory monocytes, resident monocytes and eosinophils, using the RNeasy minikit as described in Section 2.7.1. RNA of high purity and low degradation was converted to cDNA, biotin-labelled, and then hybridised to Murine Gene ST1.0 genechips (Affymetrix: Santa Clara, CA, USA). Duplicate genechips were analysed for each RNA preparation. Scanned images of labelled genechips were analysed using Partek (St. Louis, MO, USA) to give signal values and p values. The main aim of the study was to assess the association between the novel L-DC subset and well defined cells of the dendritic and myeloid lineages. Initial data analysis involved ANOVA for selection of genes up- or down-regulated by at least 3-fold in pairwise comparison. Data mining was also used to assess the expression of genes linked to known functions in development, or associated with distinct cell lineages.

Differences in overall gene expression between the subsets were revealed by Principal Component Analysis (PCA). This showed close grouping of resident monocytes, inflammatory monocytes, L-DC and cDC subsets in the first principal component, but separation of L-DC and monocyte subsets from cDC subsets in the second principal component (Figure 6.2). In addition, $CD8^+$ cDC were clearly differentiated from $CD8^-$ cDC in the second principal component. Lastly, eosoinophils were distinct from other myeloid and cDC subsets on the basis of the first and second principal components. This analysis indicated more similarity between L-DC and monocyte subsets, and differentiated L-DC from eosinophils and cDC subsets.

**Figure 6.1 Cell sorting strategy for isolation of dendritic and myeloid subsets and L-DC from spleen.**

Splenocytes were prepared by red blood cell lysis followed by T and B cell depletion. Cells were then stained with antibodies specific for CD11b (M1/70, Pe-Cy7), CD11c (N418, APC), CD8α (53-6.7, Alexa 700), Ly6C (AL-21, Pacific Blue), Ly6G (1A8, Bio), CD43 (IBII, Alexa488) and Siglec-F (E50-2440, PE), using streptavidin (SA)-APC-Cy7 as a second stage reagent. Prior to flow cytometry, cells were stained with propidium iodide (PI, 1μg/ml) to delineate live ($PI^-$) cells. Conventional DC (cDC) were initially gated on the basis of side scatter (SSC) and CD11c expression. $CD8^+$ cDC were gated as $CD11b^-CD8^+Ly6C^-Ly6G^-$ cells, while $CD8^-$ cDC were gated as $CD11b^+CD8^-Ly6C^-Ly6G^-$ cells. Myeloid cells and L-DC were further delineated on the basis of CD11b versus CD11c expression. Myeloid cells were gated as $CD11b^{hi}CD11c^-$ cells, and further delineated on the basis of Ly6C and Ly6G expression to reveal inflammatory monocytes (IM) as $Ly6C^{hi}Ly6G^-$ cells and eosinophils (Eos) as $Ly6C^+Ly6G^-CD43^+Siglec\text{-}F^+$ cells. Resident monocytes (RM) were gated as $Ly6C^+Ly6G^-CD43^+Siglec\text{-}F^-$ cells, while L-DC were gated as $Ly6C^-Ly6G^-CD43^+Siglec\text{-}F^-$ cells. Gates were set based on fluorescence minus one controls, and numbers in gates represent % specific binding.

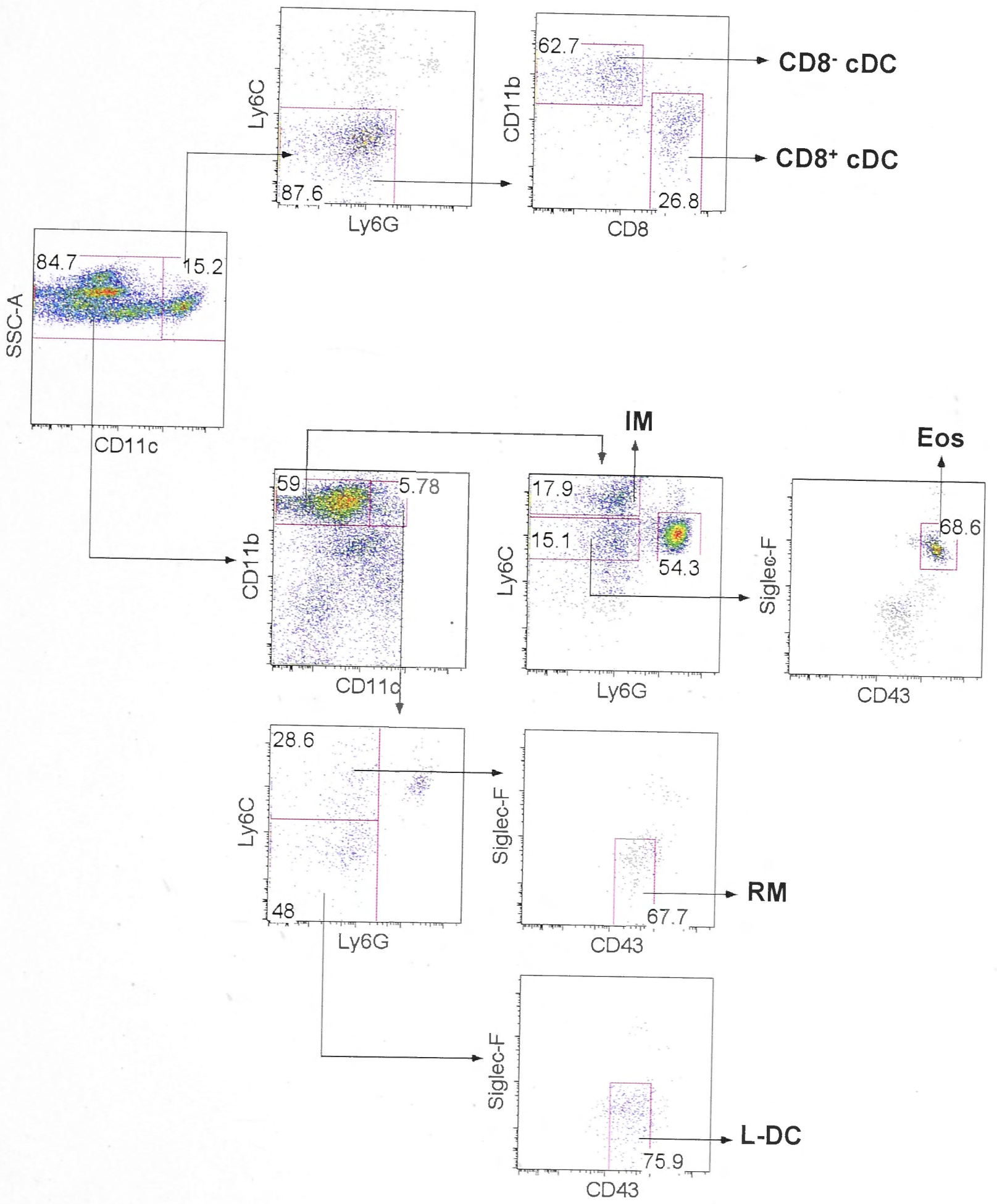
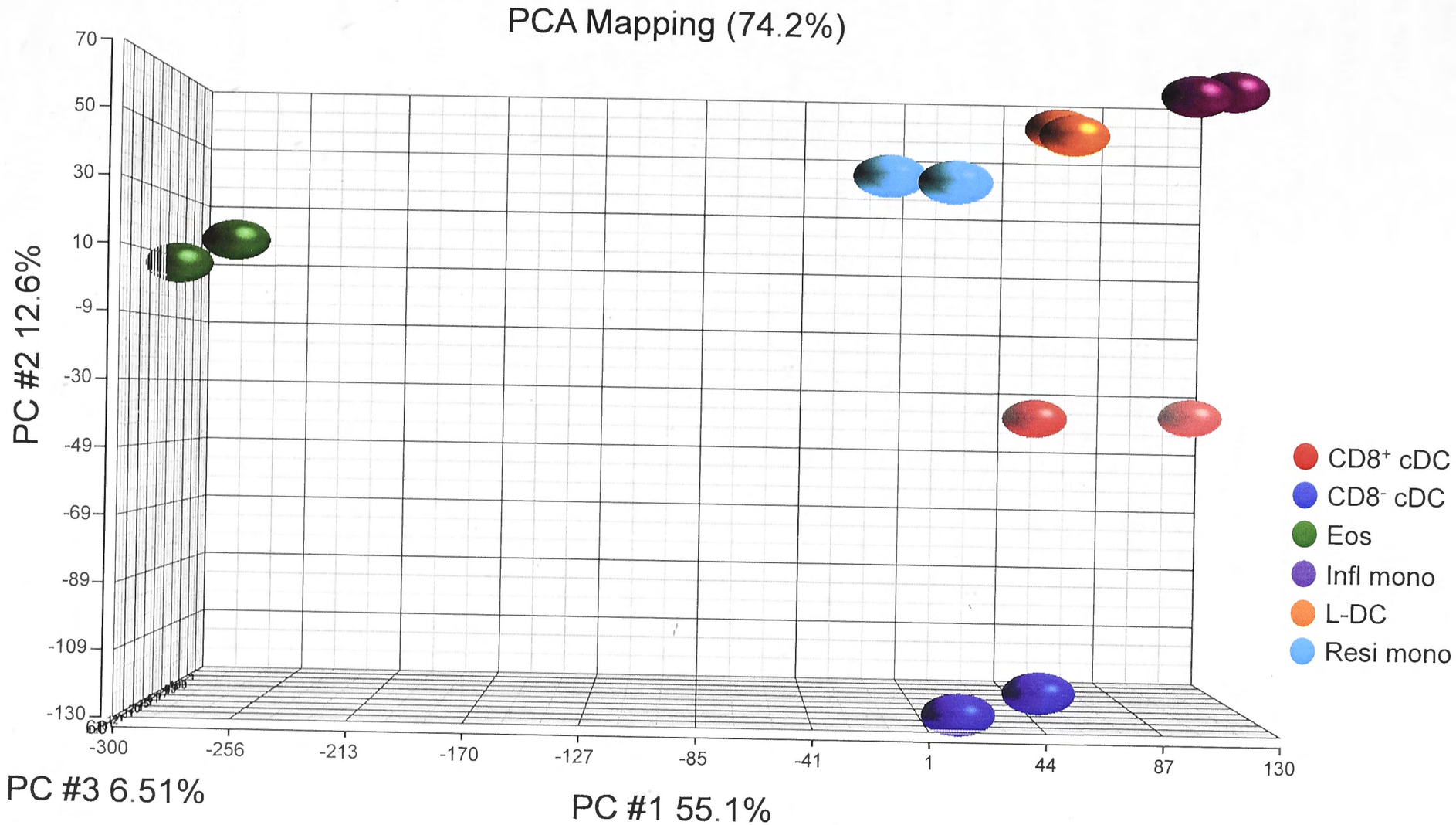

Ly6C
Ly6G
87.6
CD11b
CD8
62.7
26.8
CD8- cDC
CD8+ cDC
SSC-A
CD11c
84.7
15.2
IM
Eos
CD11b
CD11c
59
5.78
Ly6C
Ly6G
17.9
15.1
54.3
Siglec-F
CD43
68.6
Ly6C
Ly6G
28.6
48
Siglec-F
CD43
67.7
RM
Siglec-F
CD43
75.9
L-DC

**Figure 6.2 Variability in gene expression amongst dendritic and myeloid subsets.**

Transcriptome analysis was performed on subsets of cells sorted from murine spleen as described in Figure 4.3. RNA was extracted and labelled for hybridisation to Murine Gene ST1.0 genechips (Affymetrix). Following scanning to collect signal values, data were analysed using Partek and ANOVA by pairwise comparison. Principle Component Analysis (PCA) was used to determine the variability in gene expression profile for each subset. Three principle components are shown for each subset prepared for analysis in duplicate.


PCA Mapping (74.2%)
70
50
30
10
-9
-30
-49
-69
-89
-109
-130
PC #2 12.6%
-300
-256
-213
-170
-127
-85
-41
1
44
87
130
PC #3 6.51%
PC #1 55.1%
CD8+ cDC
CD8- cDC
Eos
Infl mono
L-DC
Resi mono

The pairwise relationship between subsets was demonstrated on bivariate plots (Figure 6.3 A). A total of 35,556 genes was analysed for each subset. Consistent with PCA, bivariate analysis between L-DC and resident monocytes showed the least variance, indicating high similarity in overall gene expression with very few differentially expressed genes shown as outliers (Figure 6.3 A). This was followed by L-DC and inflammatory monocytes, having higher numbers of differentially expressed genes (Figure 6.3 A). By comparison, analyses involving L-DC and cDC subsets showed more variation (Figure 6.3 A), and more differentially expressed genes. The resident and inflammatory monocyte subsets showed low variation in gene expression and very few differentially expressed genes, indicating a close relationship between these two monocyte subsets. The $CD8^+$ cDC and $CD8^-$ cDC subsets gave a tight bivariate plot indicating a close relationship, but a high number of genes differentially expressed between the subsets (Figure 6.3 A). Consistent with PCA, eosinophils showed greater differences when compared with L-DC and inflammatory monocytes (Figure 6.3). Indeed, the eosinophil subset is quite distinct from the other subsets.

Hierarchical clustering was then used to map the relationship between subsets based on gene expression. Average signal values from duplicate samples were used for clustering, and genes selected which showed expression in at least one subset (signal value $\geq 100$), giving a sample set of 8,508 genes. The analysis indicated a close relationship between L-DC and resident monocytes, and then between these two subsets and inflammatory monocytes. $CD8^+$ cDC and $CD8^-$ cDC were more closely related with each other than with L-DC, resident monocytes or inflammatory monocytes. As predicted from PCA analysis, eosinophils were shown to be quite distinct as a subset.

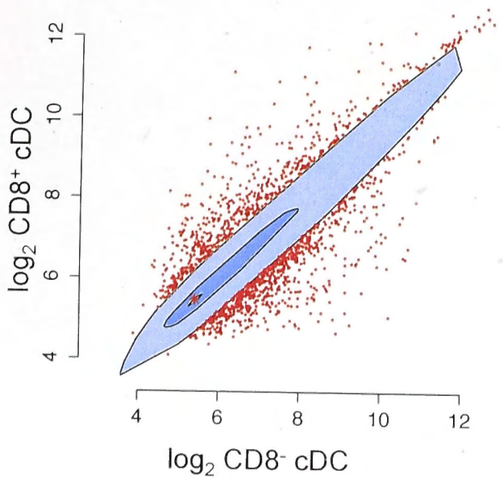
### 6.2.2 Investigation of gene expression

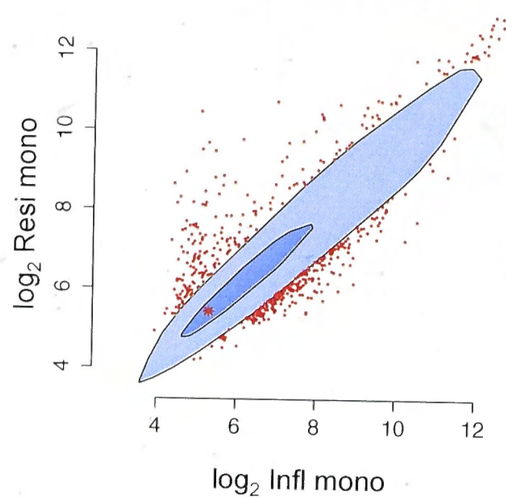
The lineage of cells in the different subsets was investigated by data mining, seeking the expression level of known genes related to the functional categories of 'DC and APC', 'Chemokines', 'Cell surface markers' and 'Inflammatory cytokines and receptors'. Signal values were collected for sets of 84 genes utilised by SABiosciences (Frederick, MD USA) in their PCR arrays. Data are shown as

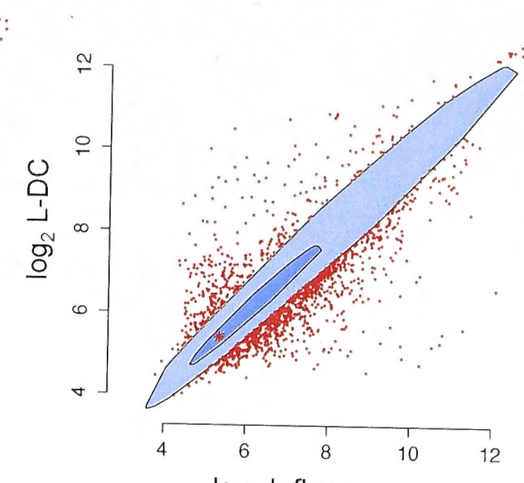
**Figure 6.3 Relationship between splenic dendritic and myeloid subsets in terms of overall gene expression.**

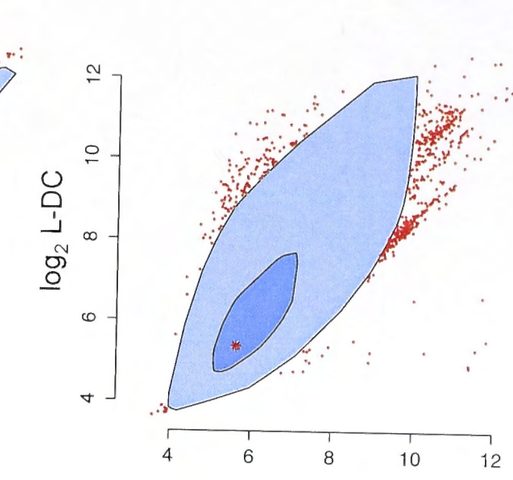
Transcriptome analysis was performed on dendritic and myeloid subsets sorted from murine spleen. Signal value data from duplicate samples were obtained using PARTEK. A) Mean signal values for a total of 35,556 genes were plotted in pairwise comparisons. The darker blue inner polygon contains 50% of data points, while the pale blue outer polygon contains all other data points which are not outliers. Red outliers are plotted outside the polygons. The bivariate median is shown by the red asterisk at the centre of the polygon. B) Hierarchical clustering was used to analyse the relationship between subsets on the basis of gene expression. The dendrogram displays distance between subsets based on clustering 8,508 genes selected for analysis on the basis of signal value $\geq 100$ for any one subset. Average signal values from duplicate samples were also used for clustering.

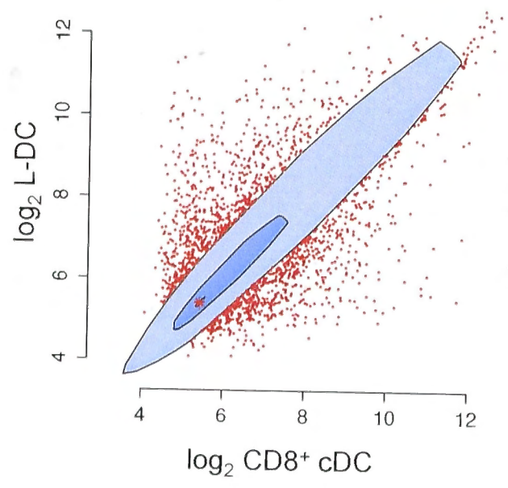
A

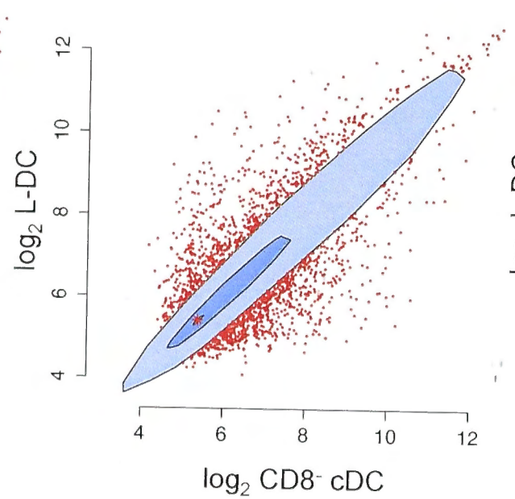

log2 CD8+ cDC
log2 CD8- cDC
4
6
8
10
12

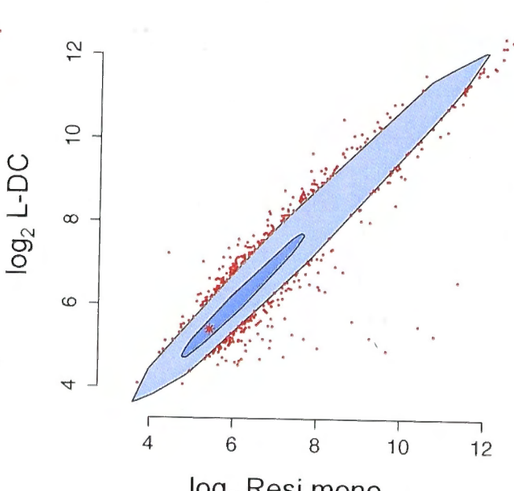

log2 Resi mono
log2 Infl mono
4
6
8
10
12

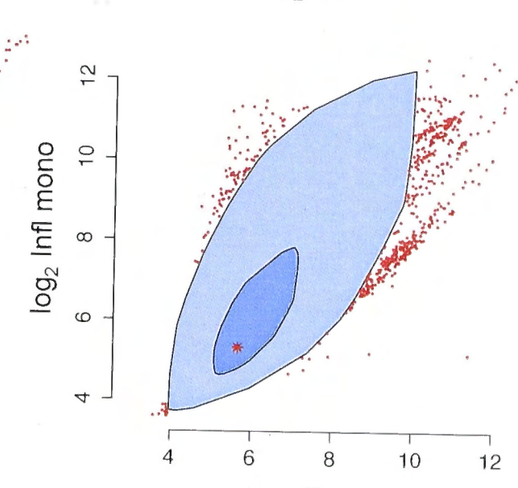

log2 L-DC
log2 Infl mono
4
6
8
10
12


log2 L-DC
log2 Eosin
4
6
8
10
12

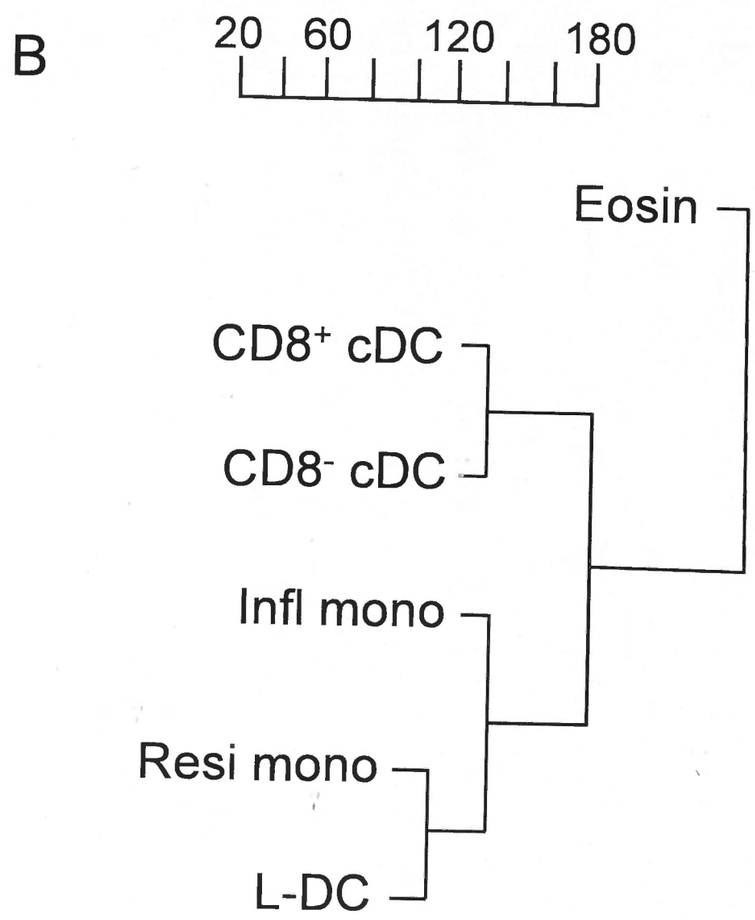

log2 L-DC
log2 CD8+ cDC
4
6
8
10
12


log2 L-DC
log2 CD8- cDC
4
6
8
10
12


log2 L-DC
log2 Resi mono
4
6
8
10
12


log2 Infl mono
log2 Eosin
4
6
8
10
12

B



heatmaps with dendrograms reflecting hierarchical clustering. Gene expression is only shown for those genes expressed by at least one subset based on signal value ≥ 100 (Figure 6.4). The $CD8^+$ cDC and $CD8^-$ cDC subsets showed greatest similarity in gene expression, and this was reflected by dendrograms in all 4 analyses (Figure 6.4 A-D). As also found in Chapter 3, these two sorted subsets were reflective of the DC lineage by common high expression of genes encoding cell surface markers including *Cd40*, *Cd74*, *Cd80*, *Cd83*, *Dpp4* and *St6gal1* (Figure 6.4 A) (Edwards et al., 2003; Lechmann et al., 2002; Miller et al., 2012; Prazma et al., 2007). Genes upregulated by $CD8^+$ cDC included *Cd8α*, *Cd24* and *Cd86*, all of which encode known markers of $CD8^+$ cDC (Belz et al., 2002; Miller et al., 2012; Vremec et al., 2000). $CD8^-$ cDC showed specific high expression of *Cd7*, *Cd22*, *Cd72* and *Klrd1*, which are also known markers of $CD8^-$ cDC (Figure 6.4 A) (Edwards et al., 2003; Miller et al., 2012).

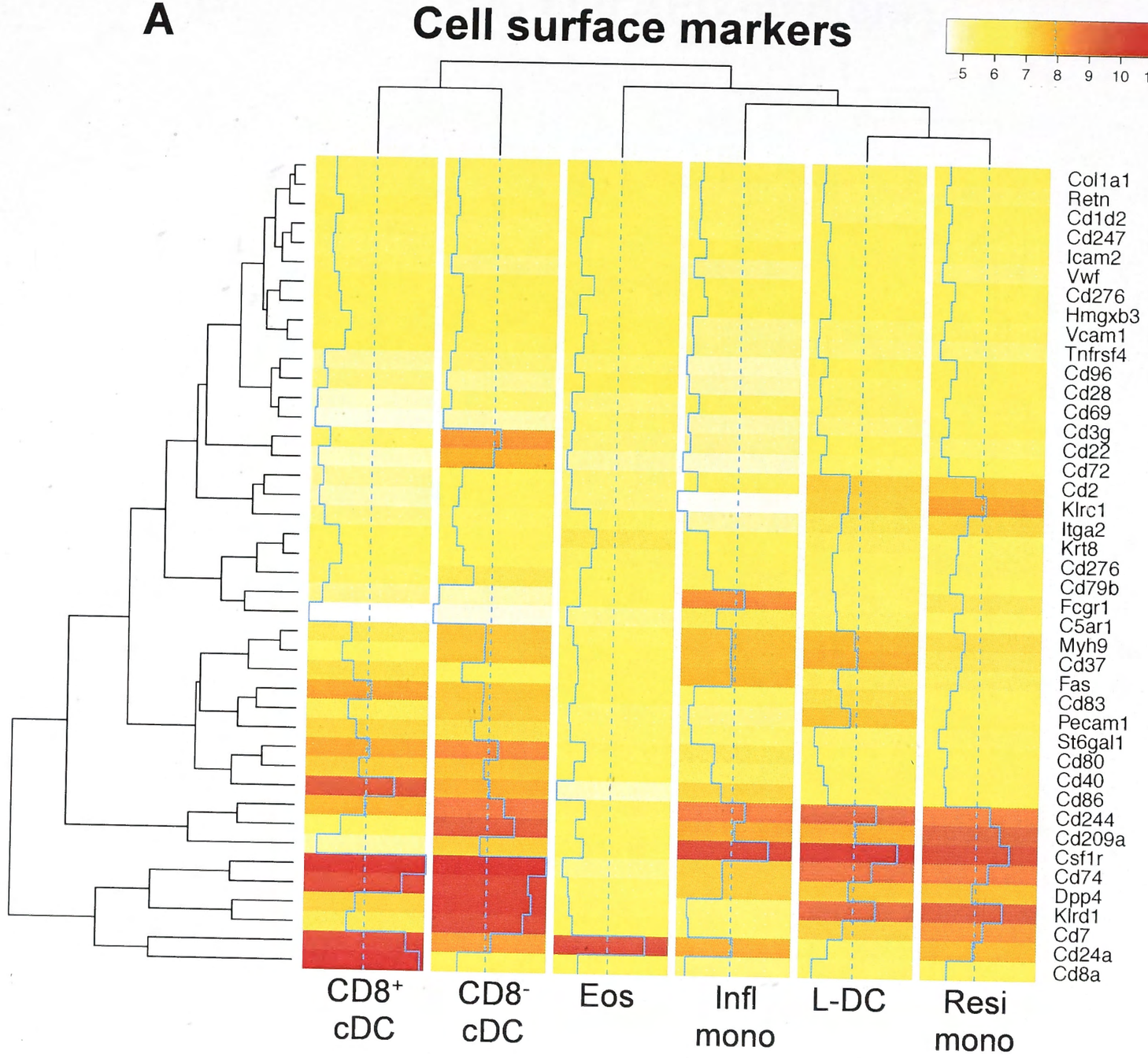
In terms of 'DC and APC' related gene expression, both cDC subsets showed high expression of *Cd74*, *Flt3*, *H2-Dma* and *Icam1*, all encoding known markers of the cDC lineage (Figure 3.7 B) (Edwards et al., 2003; Karsunky et al., 2003; Miller et al., 2012). $CD8^+$ cDC uniquely expressed *Cd86* and *Fcgrt*, while $CD8^-$ cDC showed expression of *Cd209α*, *Tlr1*, *Tnf* and *Mif*. In terms of chemokine-related genes, both cDC subsets expressed *Ccr7* and *Tnf*, which encode known chemokines and chemokine receptors expressed by DC (Allavena et al., 2000; Ding et al., 2011). $CD8^+$ cDC were uniquely marked by expression of *Xcr1* as reported previously (Bachem et al., 2010; Kroczek and Henn, 2012; Yamazaki et al., 2013), as well as expression of *Cxcl9* (Kerschen et al., 2010). $CD8^-$ cDC also specifically expressed *Ltb* (Figure 6.4 D). These data, and their concordance with descriptions of cDC gene expression in the literature, confirm the efficiency of the cell sorting procedures and gene profiling methodology developed here.

Amongst myeloid subsets, resident monocytes and inflammatory monocytes shared similar gene expression profiles for the functional categories of 'DC and APC' and 'Chemokines' (Figure 6.4 B-C). Both monocyte subsets expressed *Csf1r* which encodes the receptor for macrophage colony stimulating factor essential for monocyte development. In addition, they also expressed *Ccr2*, which encodes an essential receptor for monocyte migration (Auffray et al., 2009; Geissmann et al.,

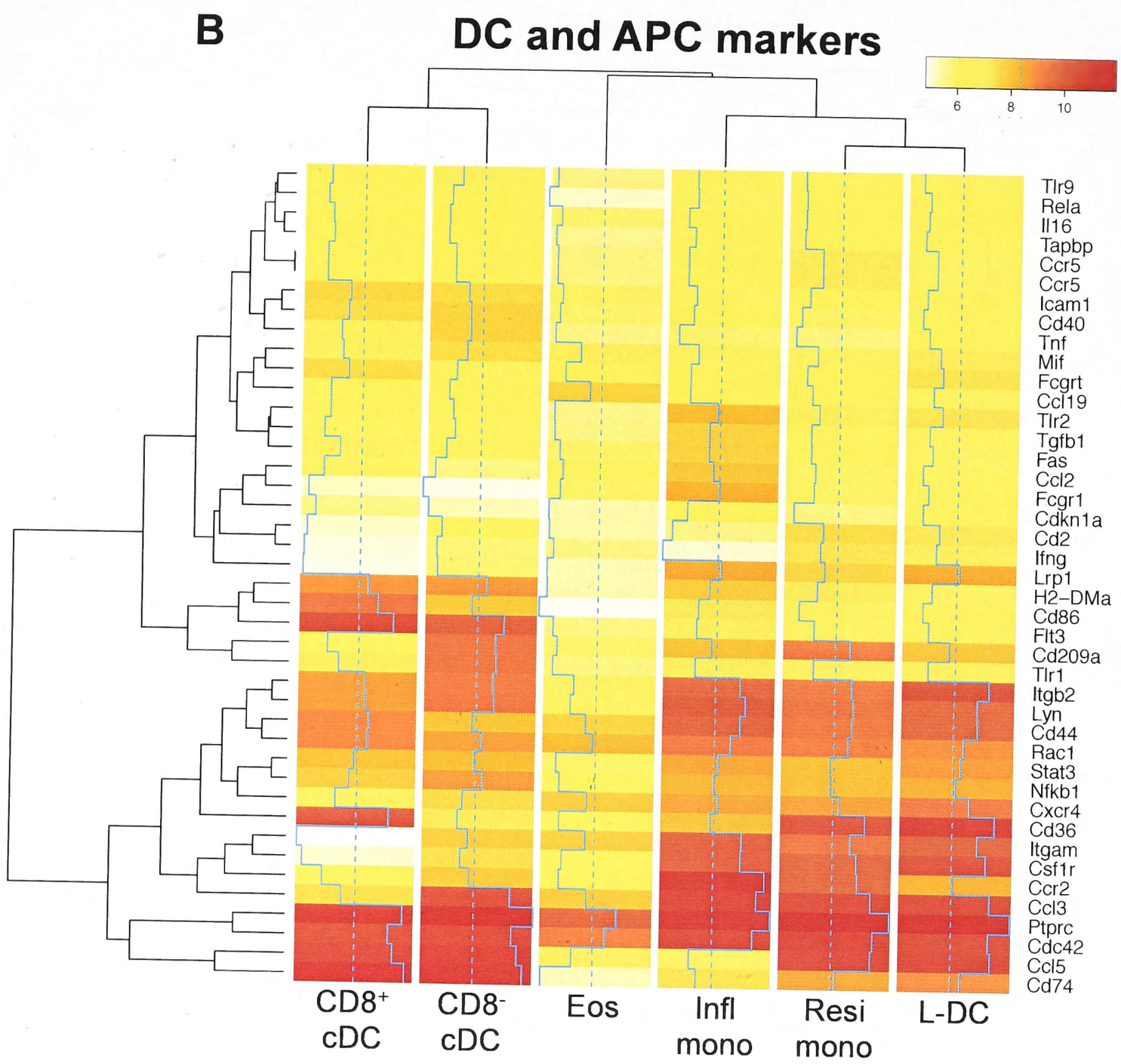
**Figure 6.4 Pathway specific gene expression in dendritic and myeloid subsets.**

Data mining was applied to Affymetrix datasets collected from L-DC, cDC and myeloid subsets. Signal values for genes selected for inclusion in specific pathways or functions were collated as Excel files. For each of the subsets, $log_2$ signal values were plotted as a heat map using R/Bioconductor software. The line chart (blue) overlaid on heat maps indicates $log_2$ signal intensity changes about the mean (dashed blue line). Genes were clustered by their level of expression as shown by row dendrograms. In addition, dendritic and myeloid subsets were clustered on the basis of gene expression as shown by column dendrograms. Data mining involved sets of genes utilised by SABioscience for their PCR arrays. These reflected : A) Cell surface markers, B) DC and APC markers, C) Chemokines, and D) Inflammatory cytokines and receptors.

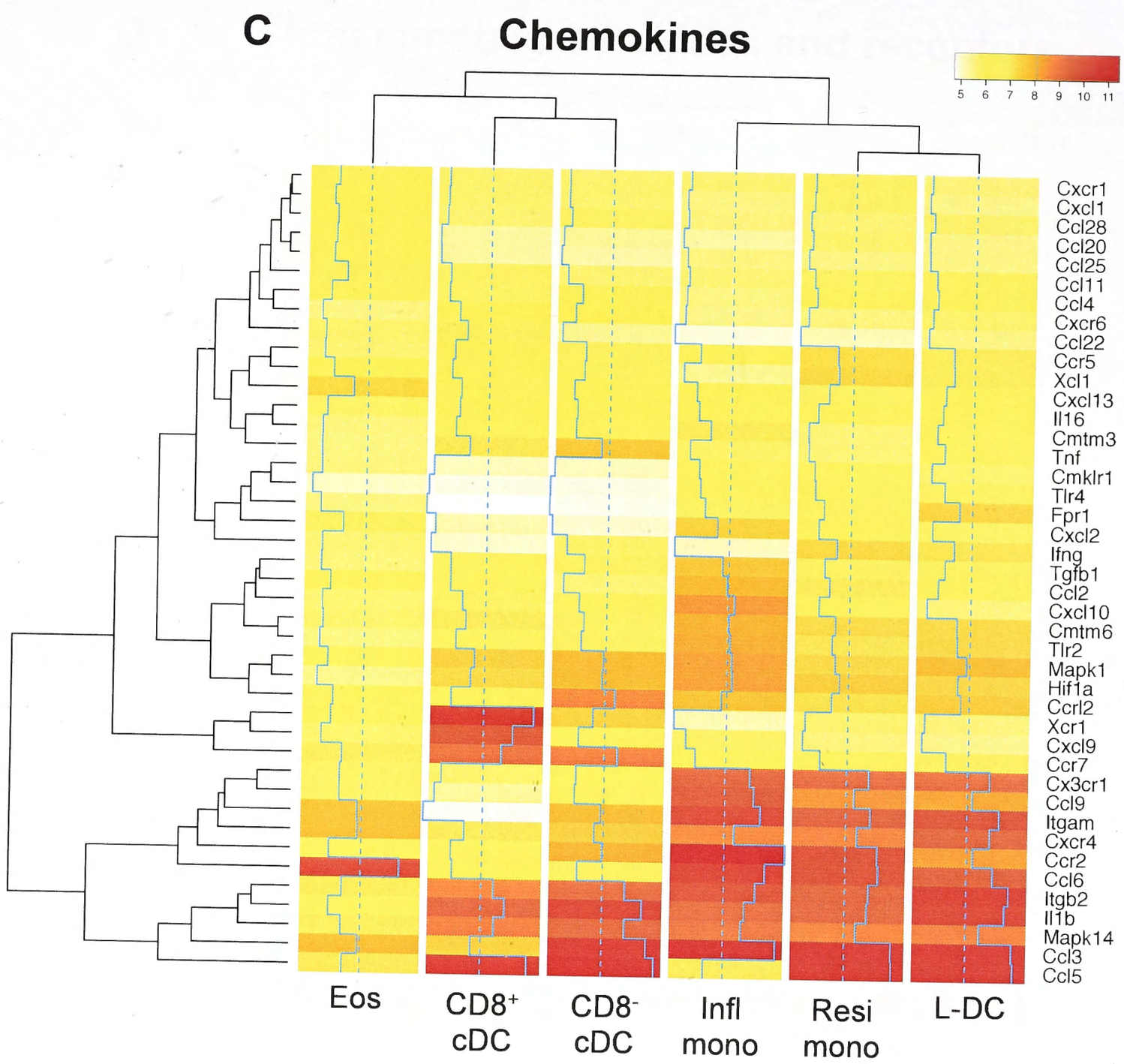
A


Cell surface markers
5 6 7 8 9 10 11
Col1a1
Retn
Cd1d2
Cd247
Icam2
Vwf
Cd276
Hmgxb3
Vcam1
Tnfrsf4
Cd96
Cd28
Cd69
Cd3g
Cd22
Cd72
Cd2
Klrc1
Itga2
Krt8
Cd276
Cd79b
Fcgr1
C5ar1
Myh9
Cd37
Fas
Cd83
Pecam1
St6gal1
Cd80
Cd40
Cd86
Cd244
Cd209a
Csf1r
Cd74
Dpp4
Klrd1
Cd7
Cd24a
Cd8a
CD8+ cDC
CD8- cDC
Eos
Infl mono
L-DC
Resi mono

**B**

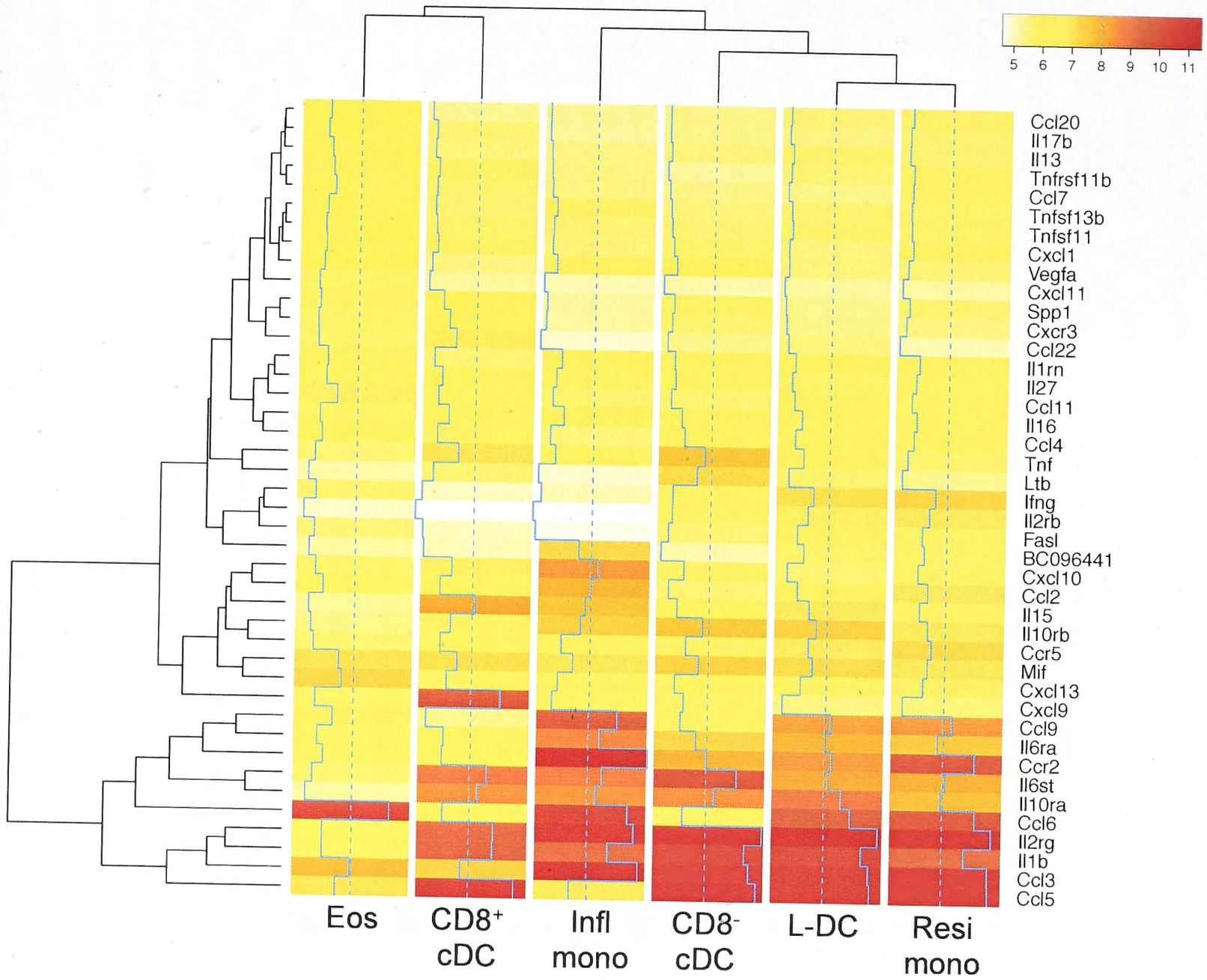

DC and APC markers
6
8
10
Tlr9
Rela
Il16
Tapbp
Ccr5
Ccr5
Icam1
Cd40
Tnf
Mif
Fcgrt
Ccl19
Tlr2
Tgfb1
Fas
Ccl2
Fcgr1
Cdkn1a
Cd2
Ifng
Lrp1
H2−DMa
Cd86
Flt3
Cd209a
Tlr1
Itgb2
Lyn
Cd44
Rac1
Stat3
Nfkb1
Cxcr4
Cd36
Itgam
Csf1r
Ccr2
Ccl3
Ptprc
Cdc42
Ccl5
Cd74
CD8+ cDC
CD8- cDC
Eos
Infl mono
Resi mono
L-DC

C


Chemokines
5 6 7 8 9 10 11
Cxcr1
Cxcl1
Ccl28
Ccl20
Ccl25
Ccl11
Ccl4
Cxcr6
Ccl22
Ccr5
Xcl1
Cxcl13
Il16
Cmtm3
Tnf
Cmklr1
Tlr4
Fpr1
Cxcl2
Ifng
Tgfb1
Ccl2
Cxcl10
Cmtm6
Tlr2
Mapk1
Hif1a
Ccrl2
Xcr1
Cxcl9
Ccr7
Cx3cr1
Ccl9
Itgam
Cxcr4
Ccr2
Ccl6
Itgb2
Il1b
Mapk14
Ccl3
Ccl5
Eos
CD8+ cDC
CD8- cDC
Infl mono
Resi mono
L-DC

D

## Inflammatory cytokines and receptors



2008). Resident monocytes specifically expressed *Ccr5*, *Il2rb*, *Klrc1* and *Ifng*, while inflammatory monocytes specifically expressed *Fcgr1*, *Fas*, *Tgfb1*, *Ccl2*, *Cxcl2*, *Cxcl10* and *Tlr2*. Consistent with PCA (Figure 6.2), eosinophils were distinct from all other myeloid and DC subsets on the basis of gene expression profiles (Figure 6.3 A-D). Eosinophils specifically expressed *Krt8*, *Ccl19* and *Cxcl13*, as reported previously (Akuthota et al., 2013; Tani et al., 2014). Since eosinophils are shown here to be very distinct from cDC and other myeloid subsets, they have been disregarded in further analysis of gene expression directed at lineage determination for L-DC.

The L-DC subset showed gene expression more closely linked with resident monocytes than with any other subsets across the four functional categories studied. This is shown by dendrograms above heatmaps (Figure 6.4 A-D). In addition, inflammatory monocytes were closely clustered with both resident monocytes and L-DC. This result is consistent with PCA and bivariate analysis, and aligns L-DC with monocytes more than cDC subsets. Genes commonly expressed in high level across L-DC and monocyte subsets included *Csf1r*, *Itgam*, *Ifng*, *Cxcr4* and *$Cx_3cr1$*. Transcriptome analysis in Chapter 3 also showed *Itgam, Csfr1 and Cxcr4* upregulation in monocyte subsets. *Itgam* encodes CD11b, a common marker of myeloid cells which mediates the inflammatory response by regulating adhesion and migration of cells to sites of infection (Arnaout et al., 1983; Solovjov et al., 2005). *$Cx_3cr1$* encodes a marker common to cells of the macrophage/dendritic lineage (Fogg et al., 2006; Jung et al., 2000; Palframan et al., 2001). L-DC also expressed *Lrp1*, *Cd37* and *Pecam1* at higher levels than seen in monocytes (Fig 6.4 A-D). LRP1 has been described as a receptor important for endocytosis of multiple functionally distinct ligands, proteases and growth factors (Lillis et al., 2008). Recent studies also showed that LRP1 regulates the signalling and migration of monocytes and macrophages (Gorovoy et al., 2010; Staudt et al., 2013). It appears to play an inhibitory role since in *$Lrp1^{-/-}$* mice recruitment of monocytes into subcutaneous tumors is significantly increased, and *$Lrp1^{-/-}$* BM-derived macrophages express higher levels of inflammatory chemokines (Staudt et al., 2013). CD37 plays a role in the regulation of cell-cell adhesion, signal transduction, and T and B cell activation (Hemler, 2001; Knobeloch et al., 2000; Van Spriel et al., 2004). Dendritic cells from *$Cd37^{-/-}$* mice are compromised in migration, as well as integrin-mediated adhesion

and *in vivo* priming of adoptively transferred naïve T cells (Gartlan et al., 2013; Van Spriel et al., 2012). As with CD37, PECAM1 (or CD31) is also involved in cell migration (Muller et al., 1993; Newman and Newman, 2003; Piali et al., 1995). The above data suggests a closer relationship between L-DC and the myeloid cell lineage than cDC lineage, although the data do not in any way establish lineage relationship. While L-DC appear more closely related to monocytes than cDC in terms of gene profile, data shown in Chapter 3 identified L-DC as having functional similarity with cDC.

Recently, Gautier et al. (2012) analysed gene expression in different tissue macrophage subsets. They defined a core signature of 39 genes defining tissue macrophages (Gautiar et al., 2012). Expression of these 39 genes was determined by data mining. However, none of the subsets studied here expressed all 39 genes, and most expressed very few (Figure A.3). Furthermore, none of the subsets analysed appears to reflect or contain a subset of tissue macrophages.

In order to test the relationship between L-DC, cDC and monocyte subsets further, core gene profiles for cDC and monocytes were sought. A list of genes common to both $CD8^+$ cDC and $CD8^-$ cDC was selected based on the criteria of signal value in both subsets $\geq 100$, and relative fold difference $\leq 1.2$. Any genes expressed by monocytes under the same criteria were also removed. A similar dataset of genes expressed by monocytes but not cDC was also produced. The expression of 'common cDC genes' and 'common monocyte genes' by L-DC was then assessed. L-DC expressed most of the common cDC genes, and their expression profile closely resembled that of both $CD8^-$ cDC and $CD8^+$ cDC subsets (Figure 6.5). However, L-DC also expressed most of the common monocyte genes, with very similar expression profiles between L-DC and both the resident and inflammatory monocyte subsets (Figure 6.6). While L-DC appear to mirror both the monocyte and cDC lineages, clustering analysis showed a closer relationship between L-DC and monocytes, than between L-DC and the cDC subsets (Figures 6.5 and 6.6).

**Figure 6.5 Genes expressed by cDC but not monocytes.**

Genes commonly expressed by $CD8^+$ cDC and $CD8^-$ cDC were selected as those with signal values $\geq 100$ in both subsets and fold change $\leq 1.2$. Genes then expressed by monocytes according to the same criteria were then removed. Expression of cDC specific genes is compared in L-DC and each of the cDC subsets. For each of the subsets, $\log_2$ signal values were plotted as a heat map using R/Bioconductor software. The line chart (blue) overlaid on heat maps indicates signal intensity changes about the mean (dashed blue line). The relationship between L-DC and cDC subsets was determined by clustering and is shown by column dendrograms.

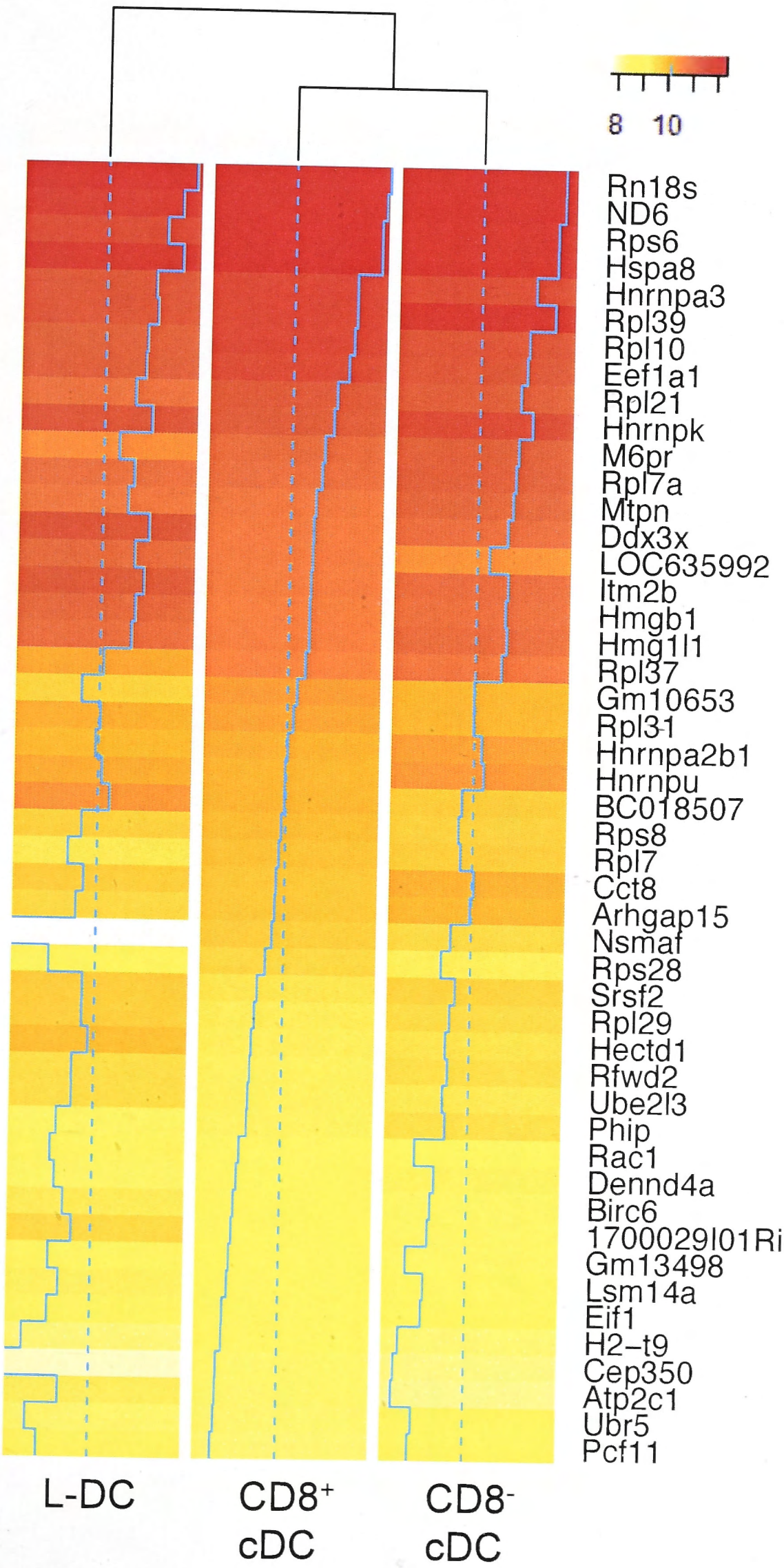
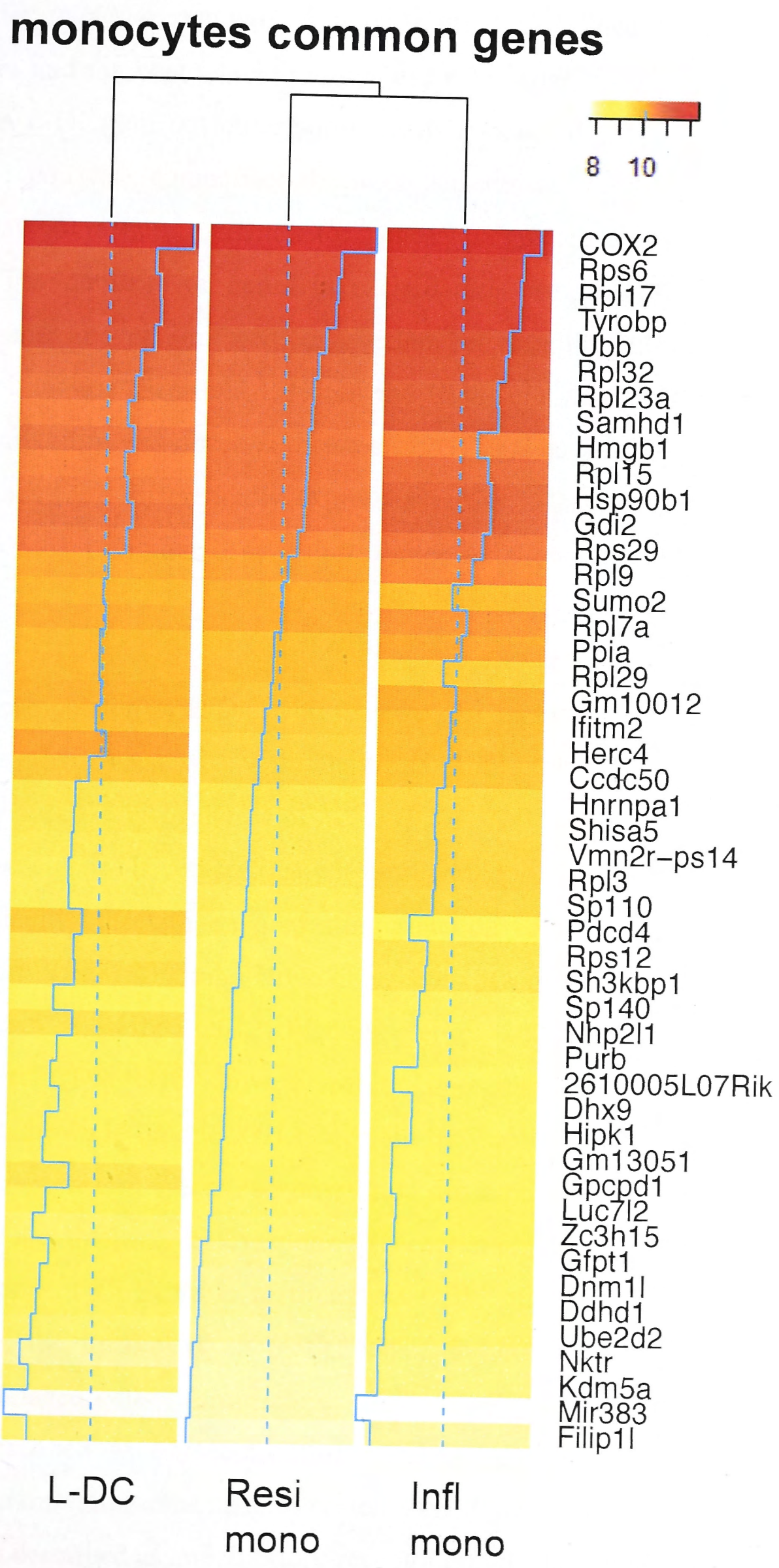

cDC common genes
8
10
Rn18s
ND6
Rps6
Hspa8
Hnrnpa3
Rpl39
Rpl10
Eef1a1
Rpl21
Hnrnpk
M6pr
Rpl7a
Mtpn
Ddx3x
LOC635992
Itm2b
Hmgb1
Hmg1l1
Rpl37
Gm10653
Rpl3-1
Hnrnpa2b1
Hnrnpu
BC018507
Rps8
Rpl7
Cct8
Arhgap15
Nsmaf
Rps28
Srsf2
Rpl29
Hectd1
Rfwd2
Ube2l3
Phip
Rac1
Dennd4a
Birc6
1700029I01Rik
Gm13498
Lsm14a
Eif1
H2–t9
Cep350
Atp2c1
Ubr5
Pcf11
L-DC
CD8+ cDC
CD8- cDC

**Figure 6.6 Genes expressed by monocytes but not cDC.**

Genes commonly expressed by inflammatory and resident monocytes were selected as those with signal values ≥100 in both subsets, and relative fold change ≤ 1.2. Genes expressed by the cDC subsets according to the same criteria were then removed. L-DC expression is shown for genes common to monocytes. Expression of monocyte genes is compared in L-DC and the two monocyte subsets. For each of the subsets, $\log_2$ signal values were plotted as heat maps using R/Bioconductor software. The line chart (blue) overlaid on heat maps indicates changes about the mean (dashed blue line). The relationship between L-DC and monocyte subsets was determined by clustering, and is shown by column dendrograms.


monocytes common genes
8
10
COX2
Rps6
Rpl17
Tyrobp
Ubb
Rpl32
Rpl23a
Samhd1
Hmgb1
Rpl15
Hsp90b1
Gdi2
Rps29
Rpl9
Sumo2
Rpl7a
Ppia
Rpl29
Gm10012
Ifitm2
Herc4
Ccdc50
Hnrnpa1
Shisa5
Vmn2r-ps14
Rpl3
Sp110
Pdcd4
Rps12
Sh3kbp1
Sp140
Nhp2l1
Purb
2610005L07Rik
Dhx9
Hipk1
Gm13051
Gpcpd1
Luc7l2
Zc3h15
Gfpt1
Dnm1l
Ddhd1
Ube2d2
Nktr
Kdm5a
Mir383
Filip1l
L-DC
Resi mono
Infl mono

### 6.2.3 Genes upregulated in L-DC over the cDC subsets

In order to advance understanding of the L-DC lineage, and to identify potential genes and markers which define these cells, analysis of genes specifically upregulated in L-DC, but not other subsets was conducted. ANOVA analysis was used to make pairwise comparison between subsets as described previously in Chapter 3. Data were then extracted for genes upregulated at least 3-fold in one subset only. The numbers of genes in each dataset was represented in a Venn diagram as a measure of similarity or overlap between the subsets (Figure 6.7). Lowest variability was identified between the $CD8^+$ cDC and $CD8^-$ cDC subsets, while larger variability was observed between L-DC and each of the cDC subsets. A total of 24 genes were differentially expressed across L-DC, $CD8^+$ cDC and $CD8^-$ cDC (Figure 6.7 A). Complete lists of these genes are shown in Tables A.1, A.2 and A.3.

In the comparison of L-DC with the two cDC subsets, genes upregulated in one but not the other two subsets were identified (Table 6.1). The $CD8^+$ cDC subset was marked by specific upregulation of *Xcr1*, *Ifi205*, *Tlr11*, *Btla*, *CD24a* and *Clec9a* (Table 6.1). These genes have previously been reported as specific markers for $CD8^+$ cDC, which identify the antigen presenting function of a DC (Caminschi et al., 2001a; Caminschi et al., 2008; del Hoyo et al., 2002; Galibert et al., 2005; Ghosh et al., 2013; Kroczek and Henn, 2012; Watanabe et al., 2003). Analysis in Chapter 3 also showed that $CD8^+$ cDC showed specific upregulation of *Xcr1* and *Clec9a*. XCR1 has been shown to bind to XCL1 secreted by T cells and aids the migration of DC to T cell areas within spleen, so promoting cross-priming of $CD8^+$ T cells for development of a cytotoxic T cell response (Dorner et al., 2009; Kroczek and Henn, 2012). Expression of CLEC9a is restricted to $CD8^+$ cDC and pDC, and plays a specific role in the uptake of apoptotic cells via binding to the exposed actin filaments of damaged cells (Caminschi et al., 2008; Zhang et al., 2012). Recently IFI205 was described as a receptor that regulates signalling via transcriptional regulation of the inflammasome adapter protein ASC (Ghosh et al., 2013). BTLA has previously been described as an inhibitory receptor on DC that regulates T and B cell activation (Carreno and Collins, 2003). BTLA can also induce $CD8^+$ T cell tolerance in NOD mice, and acts as a regulator of DC homeostasis (De Trez et al., 2008; Li et

**Figure 6.7 Differential gene expression.**

Diagrams show numbers of genes upregulated ≥ 3-fold in one of two subsets assessed in pairwise comparison. Infl mono: Inflammatory monocytes; Resi mono: Resident monocytes.

A


CD8+ cDC
CD8- cDC
288
47
57
160
24
113
252
244
L-DC
CD8- cDC
446
L-DC
CD8+ cDC
680

B

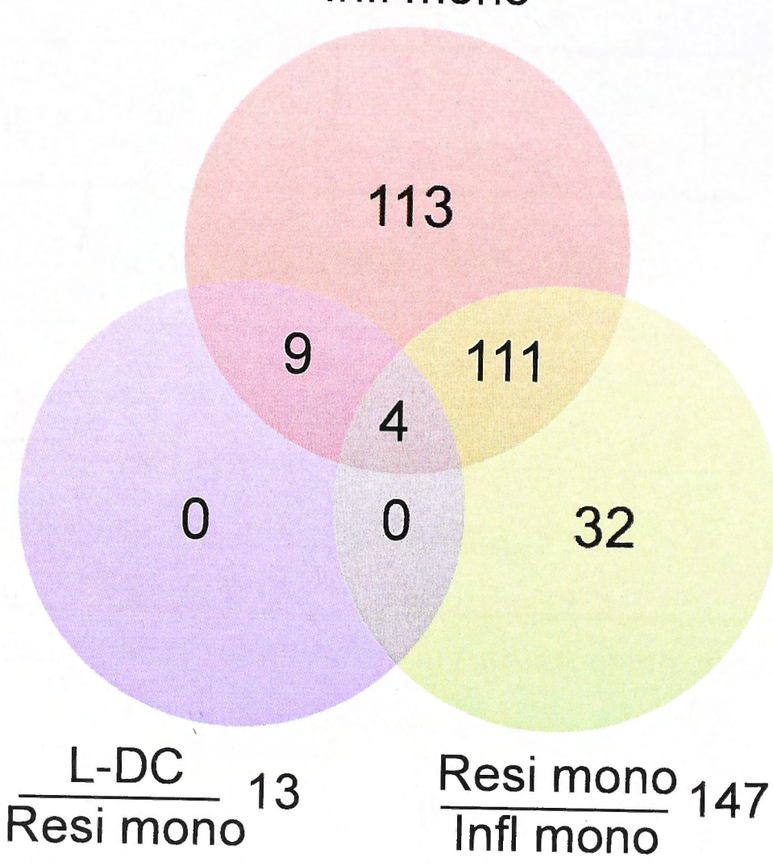

L-DC
Infl mono
237
113
9
111
4
0
0
32
L-DC
Resi mono
13
Resi mono
Infl mono
147

**Table 6.1 Genes upregulated in only one of three DC subsets.**

| Subset | No. of genes | Genes upregulated [+] |
|---|---|---|
| $CD8^+$ cDC | 9 | *CD24a, Ifi205, Clec9a, Tlr11, Xcr1, Apol7c, Btla, A530099J19Rik, 4930506M07Rik* |
| $CD8^-$ cDC | 2 | *Ccnd1, H2-Eb2* |
| L-DC | 13 | *Tlr7, Ctsd, Itgam, Clec4a3, Zeb2, Csf1r, Emr4, Krt80, Cd300ld* |

[+] Genes were selected which showed ≥ 3-fold difference in signal value across each of three subsets taken in pairwise comparison. Individual subsets are distinguished by specifically upregulated genes.

al., 2011). Genes upregulated by $CD8^-$ cDC included only *Ccnd1* and *H2-Eb2*. CCND1 is involved in cell cycle progression, while H2-Eb2, also known as MHCII, is expressed by APC (Shin et al., 2006; Sun et al., 2008).

By comparison with the cDC subsets, L-DC showed upregulation of genes which reflect both dendritic and myeloid lineage cells. *Clec4a4*, also known as *Dcir2*, encodes a C-type lectin previously described for $CD8^-$ cDC. CLEC4a4 participates in multiple pathways depending on its immunoreceptor (Dudziak et al., 2007; Idoyaga et al., 2013). *Emr4* encodes the epidermal growth factor receptor on $CD8^-$ cDC, monocytes and some macrophages (Caminschi et al., 2001b). In addition, L-DC express higher levels of *Zeb2* and *Krt80*, which are involved in adhesion and migration (Qi et al., 2012; Windoffer et al., 2011), as well as myeloid cell markers like *Itgam* and *Csf1r* as found in Chapter 3. L-DC also upregulated *Cd300ld* in relation to cDC subsets. *Cd300ld* encodes a type I transmembrane protein with a short cytoplasmic tail and a charged transmembrane residue (Borrego, 2013). CD300LD is expressed by granulocytes, monocytes, macrophages, monocyte-derived DC (mo-DC) and pDC (Izawa et al., 2007), and interacts with adaptor protein FcRγ chain to transmit an activation signal via LYN and SYK kinases (Izawa et al., 2007). Upon binding to cells, CD300LD induces increased production of cytokines, particularly IL-6, and histamine release from bone marrow-derived mast cells (Izawa et al., 2007). The binding of CD300LD to ligands on macrophages and neutrophils also induces increased pro-inflammatory cytokine production (Nakano et al., 2008).

### 6.2.4 Genes upregulated in L-DC but not the monocyte subsets

Datasets were extracted to identify genes upregulated at least 3-fold in either L-DC or the 2 monocytes subsets. L-DC and inflammatory monocytes were found to be the most distinct subsets, while L-DC and resident monocytes were the most closely related (Figure 6.5 B). These findings are consistent with previous PCA and clustering evidence (Figures 6.2-6.4). Only 4 genes were found to be commonly up- or down-regulated in L-DC compared with the two monocyte subsets.

In the comparison of gene expression between L-DC, resident monocytes and inflammatory monocytes, only L-DC and inflammatory monocytes were found to show upregulated genes. Resident monocytes therefore reflect a subset of both the L-DC and inflammatory monocyte subsets (Table 6.2). Only small numbers of genes were upregulated. *Fn1*, *F13a1*, and *Mmp8* identified inflammatory monocytes, and upregulation of *Cd300e* identified L-DC. All of this evidence is consistent with previous data showing that resident monocytes are closely related to L-DC. It also predicts a close relationship between resident monocytes and inflammatory monocytes. Previously in Chapter 3, inflammatory monocytes were shown to upregulate *F13a1* which encodes an alternate activation marker for macrophages (Table 3.4) (Martinez et al., 2006; Muszbek et al., 1996). In addition, inflammatory monocytes showed upregulation of *Fn1* which encodes fibronectin1, and *Mmp8* which encodes matrix metalloproteinase-8. FN1 is involved in cell adhesion, migration and growth, while MMP8 is a protease involved in breaking down extracellular matrix. In addition, FN1, F13A1 and MMP8 have also been previously described as specifically upregulated in inflammatory monocytes over resident monocytes by others (Ingersoll et al., 2010).

L-DC upregulated only *Cd300e*, as previously identified in Chapter 3. CD300E is a type I transmembrane protein with a short cytoplasmic tail and a charged transmembrane residue which interacts with DAP12 (Borrego, 2013). It is expressed mainly by monocytes, mo-DC, and at lower levels by *in vitro* derived macrophages and DC (Borrego, 2013). Upon binding, CD300E induces activation signals, calcium mobilisation and release of reactive oxygen species by monocytes (Aguilar et al., 2004; Brckalo et al., 2010). In addition, CD300E binding also induces cytokine release by monocytes and promotes survival of monocytes and mo-DC (Aguilar et al., 2004; Brckalo et al., 2010). DC activated via CD300E have stronger capacity to stimulate T cells (Brckalo et al., 2010). Overall, CD300E reflects a possible candidate marker for delineation of L-DC from the two monocyte subsets.

### 6.2.5 Identification of genes which distinguish L-DC from resident monocytes

Both PCA and gene expression analyses revealed a close developmental relationship between resident monocytes as $CD11b^{hi}CD11c^{+}Ly6C^{+}Ly6G^{-}$

**Table 6.2 Genes upregulated in only one of three myeloid subsets.**

| Subset | No. of genes | Genes upregulated [+] |
|---|---|---|
| Inflammatory monocytes | 3 | *Fn1, F13a1, Mmp8* |
| Resident monocytes | 0 | nil |
| L-DC | 1 | *Cd300e* |

[+] Genes were selected which showed ≥ 3-fold difference in signal value across each of three subsets taken in pairwise comparison. Individual subsets are distinguished by specifically upregulated genes.

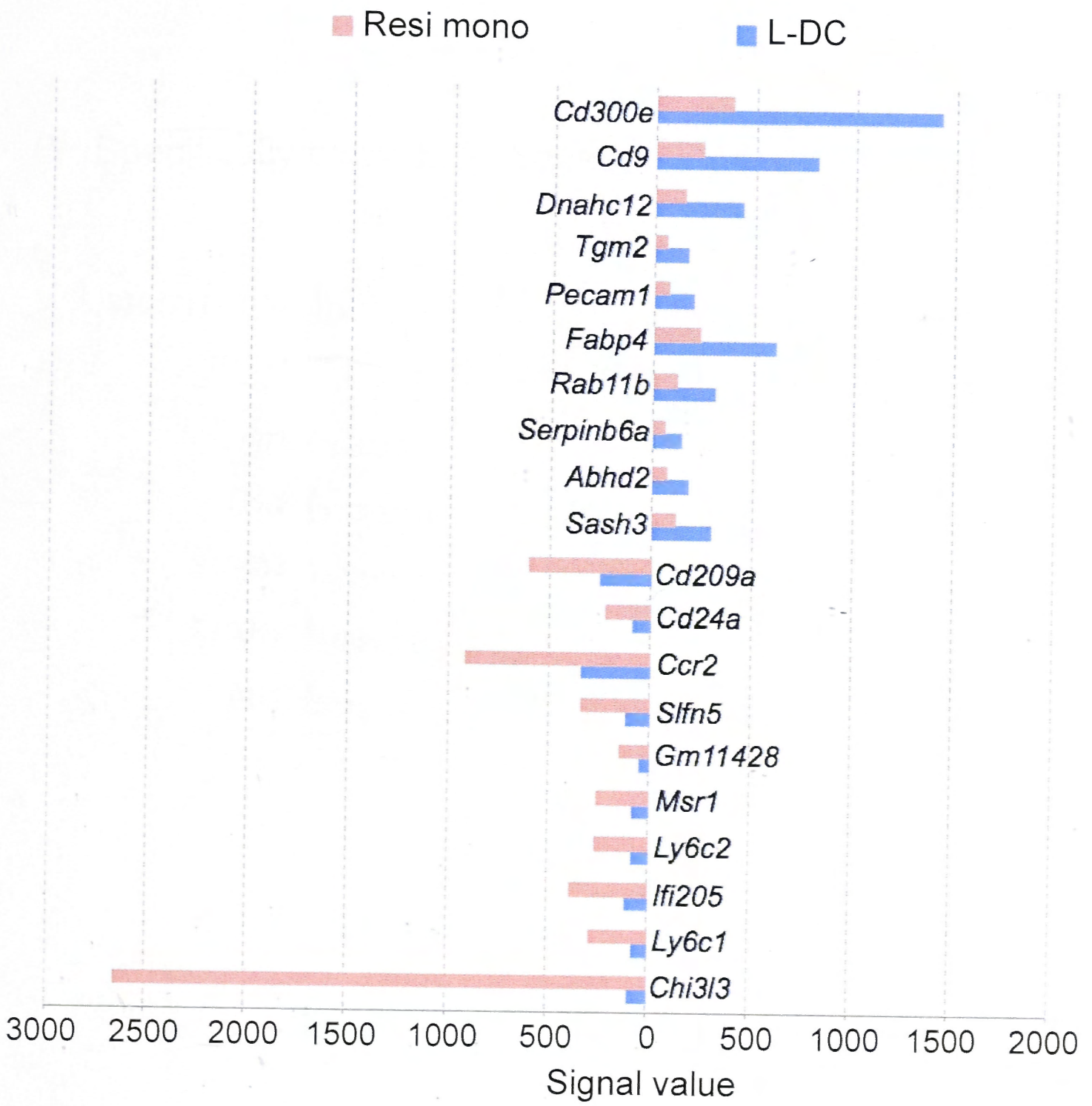
$CD43^{+}Siglec\text{-}F^{-}$ cells and L-DC which differ mainly by the low levels of CD43 and absence of Ly6C expression. To further investigate this relationship, genes specifically upregulated in either L-DC or resident monocytes were identified (Figure 6.8 A). In line with previous analyses (Table 6.2), *Cd300e* was found to be upregulated in L-DC over resident monocytes, along with *Cd9, Dnahc12, Tgm2, Pecam1, Fabp4, Rab11, Serpinb6a, Abhd2* and *Sash3*. Both CD300E and CD9 regulate the ability of DC to activate T cells (Aguilar et al., 2004; Brckalo et al., 2010; Kobayashi et al., 2004). In addition, CD9 also modulates cell adhesion and migration (Lagaudrière-Gesbert et al., 1997), and acts as a potent co-stimulatory molecule for T cells (Kobayashi et al., 2004). DNAHC12 belongs to the dynein family, comprising proteins that convert energy in ATP into energy in movement (Roberts et al., 2013), while FABP4 is involved in T cell priming via regulation of IFN-γ production by $CD8^{+}$ T cells (Elmasri et al., 2009; Gorbenko et al., 2006; Roberts et al., 2013). TGM2 is involved in multiple processes including apoptosis and signal transduction (Rébé et al., 2009). RAB11B has been found to participate in both endocytic and exocytic pathways involving Fc receptors to transport intracellular antigens (Schlierf et al., 2000; Ward et al., 2005). SERPINB6A is essential for protecting $CD8^{+}$ T cytotoxic lymphocytes against the action of its' own cytotoxic granules (Zhang et al., 2006). SASH3, also known as SLY1, participates in the regulation of marginal zone B cell development via the Notch signalling pathway (Scheikl et al., 2009). All of these genes reflect the function of antigen presenting cells, consistent with the defined functional role of L-DC in $CD8^{+}$ T cell activation in Chapter 5.

Consistent with reports in the literature, resident monocytes expressed gene encoding the known markers of LY6C1, LY6C2, and CCR2 (Figure 6.8) (Auffray et al., 2009; Geissmann et al., 2008). Expression of Ly6C1/2 reflects the adopted sorting strategy used to separate resident monocytes from L-DC on the basis of this marker (Figure 4.7). In addition, resident monocytes also showed upregulation of genes expressed by macrophages including *Chi3l3, Ifi205, Msr1, Amwap* and *Cd209a* (Figure 6.8 A) (Gundra et al., 2014; Ingersoll et al., 2010; Nio et al., 2012; Platt and Gordon, 2001). *Chi3l3* is clearly upregulated by resident monocytes (Figure 6.8). Expression of *Ifi205* has been described for both DC and macrophages, where it regulates the inflammasome adapter protein ASC (Ghosh et al., 2013; Inglis et al.,

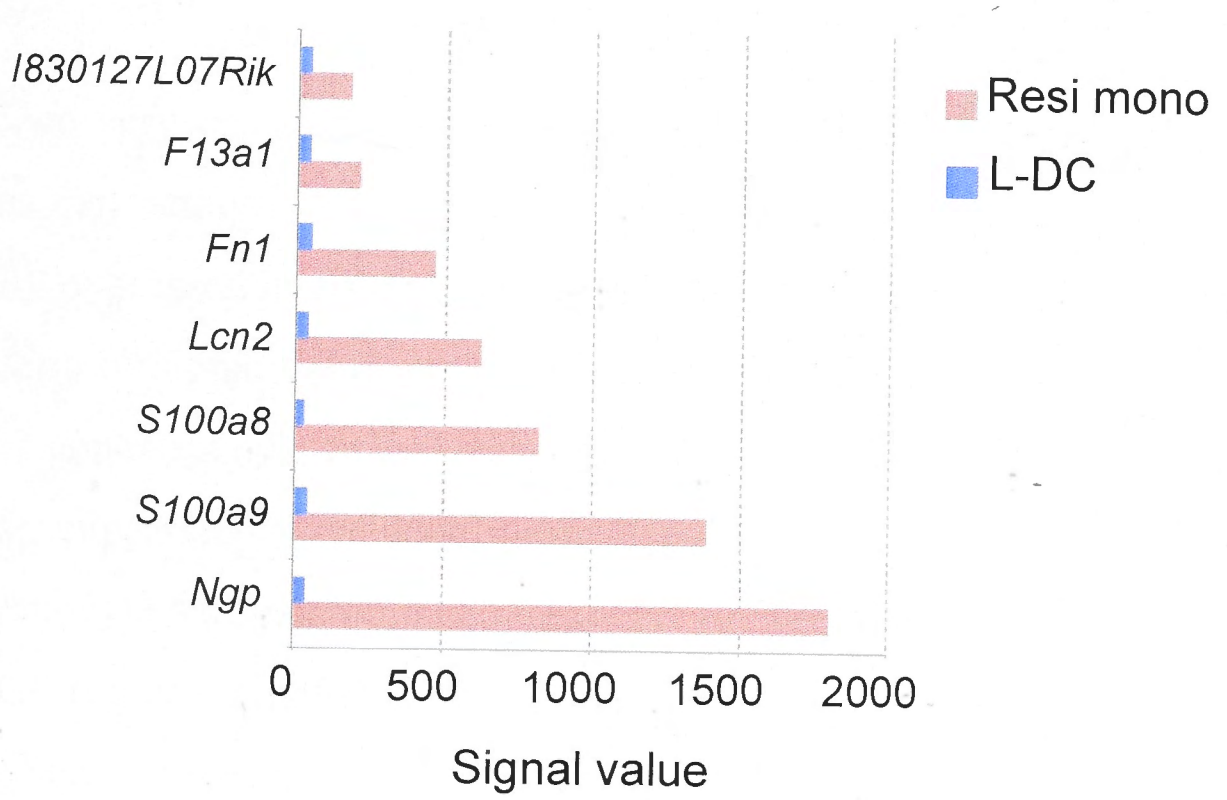
**Figure 6.8 Genes upregulated or specifically expressed between L-DC and resident monocytes.**

ANOVA analysis was used to make pairwise comparisons of gene expression between samples and to calculate relative fold changes. A) Genes upregulated in either L-DC or resident monocytes were selected as those for which the signal value in one subset was $\geq 50$, and the signal value in the second subset was $\geq 125$. Data shown reflect genes with $\geq 2.5$ fold difference in signal value. B) Genes specifically expressed in either L-DC or resident monocytes were selected as those for which the signal value in one subset was $\leq 50$, and the signal value in the second subset was $\geq 125$. This gave a dataset of 7 genes, with none specifically expressed by L-DC.

A Upregulated genes



B Specifically expressed genes



2010; Orlova et al., 2006). *Msr1* is expressed by both macrophages and DC, and is involved in the endocytosis of double stranded RNA, transportation to endosomes, and interaction with TLR3 for triggering IFN responses (Dansako et al., 2013; Herber et al., 2010). As with MSR1 and IFI205, CD209a is also expressed on both macrophages and DC (Cheong et al., 2010; Taylor et al., 2005). CD209a recognises and binds mannose-type carbohydrates found on viruses, bacteria and fungi, and induces phagocytosis of pathogens by macrophages (McGreal et al., 2005). AMWAP is expressed by tissue macrophages, microglia and retinal cells, and regulates proinflammatory microglia and macrophage activation (Karlstetter et al., 2010). The list of genes described above confirms that the sorted resident monocyte population isolated here accurately reflects the subset described in the literature.

Since very few genes were identified as upregulated by L-DC over resident monocytes, an analysis of specifically expressed genes was conducted. Genes specifically expressed in either L-DC or resident monocytes were selected according to the criteria of signal value $\geq 125$ in one subset and $\leq 50$ in the other. This gave a subset of 7 genes specific to resident monocytes, but none for L-DC. Amongst genes specifically expressed by resident monocytes, *Ngp*, *S100a8* and *S100a9* are known to encode monocyte and macrophage markers (Ehrchen et al., 2009; Schiopu and Cotoi, 2013). NGP regulates monocyte functions of activation and recruitment into sites of infection (Soehnlein et al., 2009). Both S100A8 and S100A9 have been described as activators of endogenous TLR4, so promoting proinflammatory responses (Ehrchen et al., 2009; Schiopu and Cotoi, 2013). LCN2 is expressed by neutrophils and limits bacterial growth via sequestration of bacterial siderophores containing iron (Flo et al., 2004). Lastly, both *Fn1* and *F13a1* are upregulated in inflammatory monocytes over resident monocytes (Table 6.2). Resident monocytes have higher expression of these markers over L-DC, but lower expression compared with inflammatory monocytes.

Gene expression analysis has therefore enabled further distinction between resident monocytes and L-DC. Overall, L-DC show upregulation of many genes with functional roles in antigen processing and presentation to T cells, while resident monocytes show upregulation of genes previously described in relation to monocyte and macrophage function. This evidence supports previous studies showing

distinction in terms of marker expression, morphology and capacity to activate T cells.

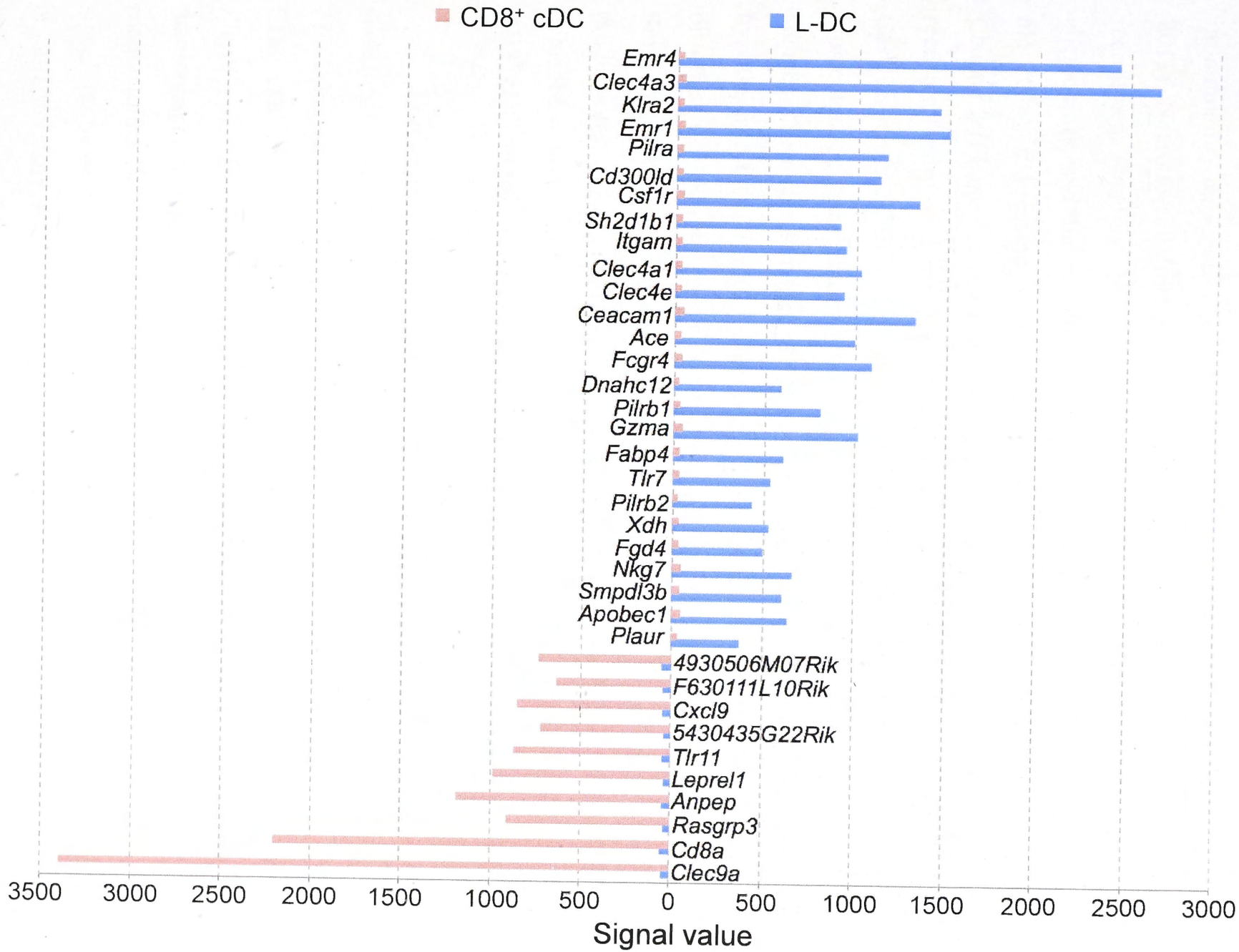
### 6.2.6 Further attempts to identify specific markers of L-DC

The combination of phenotypic, functional and genomic evidence suggests that L-DC reflect a myeloid lineage DC. To further investigate the relationship between L-DC and cDC, specifically expressed genes in either L-DC or each of the cDC subsets were identified. In pairwise comparison between L-DC and $CD8^+$ cDC, L-DC were found to specifically express multiple genes related to myeloid cells (Figure 6.9). In line with the literature, L-DC expressed *Itgam* (CD11b) which is not expressed by $CD8^+$ cDC (Tan et al., 2011). In addition, L-DC express *Klra2* also known as *Ly49b*, which is expressed by monocytes, macrophages, NK cells and DC (Gays et al., 2006; Yamada and Tsuchida, 2014). Ly49B interacts with SHP-1, SHP-2, and SHIP to regulate signalling events (Gays et al., 2006). In addition, Ly49B regulates T cell differentiation via recognition of MHCI molecules on T cells (Yamada and Tsuchida, 2014). L-DC also specifically expressed the *Pilrα*, *Pilrβ1* and *Pilrβ2* genes which encode proteins that regulate SHP signalling. SHP plays a central role in multiple cell signalling pathways via dephosphorylation of tyrosine residues. The balance of PILRα-mediated inhibitory signals and PILRβ-mediated activation signals regulates signalling via SHP, although the exact functions of PILRα and PILRβ are unknown. PILRα was previously shown to be important for neutrophil recruitment during inflammation (Wang et al., 2013), while PILRβ mediates an inflammatory response via production of IL-27 by antigen presenting cells and IL-10 by effector T cells (Tato et al., 2012). L-DC also specifically expressed genes related to macrophages including *Emr4*, *Emr1* and *Csf1r* (Figure 6.9) (Corbett et al., 2005; Geissmann et al., 2008; Stacey et al., 2002). L-DC also showed specific expression of *Nkg7* and *GzmaA* (Figure 6.9). Both genes encode granzymes involved in the induction of apoptosis. In addition, expression of NKG7 has been described for neutrophils and NK cells (Shimane et al., 1999).

L-DC also specifically expressed multiple genes related to DC although not $CD8^+$ cDC, including *Clec4a3*, *Clec4a1*, *Clec4e*, *Ace*, *Fabp4* and *CD300ld* (Figure 6.9). CLEC4 represents a family of transmembrane C type lectin receptors involved

**Figure 6.9 Genes specifically expressed in either $CD8^+$ cDC or L-DC.**

ANOVA analysis was used to make pairwise comparisons of gene expression between the $CD8^+$ cDC and L-DC subsets. Genes specifically expressed in either $CD8^+$ cDC or L-DC were selected based on the criteria of signal value in one subset $\leq 50$, and signal value in the other $\geq 150$. This gave a dataset of 139 genes (See Table A.4). Only those showing $\geq 15$ fold difference in signal value are shown.


CD8⁺ cDC
L-DC
Emr4
Clec4a3
Klra2
Emr1
Pilra
Cd300ld
Csf1r
Sh2d1b1
Itgam
Clec4a1
Clec4e
Ceacam1
Ace
Fcgr4
Dnahc12
Pilrb1
Gzma
Fabp4
Tlr7
Pilrb2
Xdh
Fgd4
Nkg7
Smpdl3b
Apobec1
Plaur
4930506M07Rik
F630111L10Rik
Cxcl9
5430435G22Rik
Tlr11
Leprel1
Anpep
Rasgrp3
Cd8a
Clec9a
3500
3000
2500
2000
1500
1000
500
0
500
1000
1500
2000
2500
3000
Signal value

in diverse functions, including cell adhesion, signalling and inflammation. L-DC expressed 2 isoforms of CLEC4A, described for $CD8^-$ cDC (Dudziak et al., 2007; Idoyaga et al., 2013). CLEC4E functions in antigen presentation and initiation of inflammatory responses after cell death (Barr et al., 2007; Osorio and Reis e Sousa, 2011). Necrotic cells release SAP-130 which binds to CLEC4E, initiating caspase recruitment leading to the production of pro-inflammatory cytokines and chemokines, and the recruitment of phagocytes to inflammatory sites (Yamasaki et al., 2008). Expression of *Ace* was also observed in L-DC and was identified in Chapter 3 (Figure 6.9 and Table 3.5). CD300LD is a member of the CD300 immune receptor family, and has been described on human myeloid lineage DC (Comas-Casellas et al., 2012; Gasiorowski et al., 2013). When cross-linked, CD300 molecules regulate leukocyte function by transmitting activating and/or inhibitory signals. The exact function of CD300LD remains unknown, however, it has been hypothesised that CD300LD may regulate the expression of CD300F, and the level of other CD300 molecules on the cell surface (Comas-Casellas et al., 2012). Similarly, FABP4 has also been described for human mo-DC. It is involved in production of IL-12 and TNF, and T cell priming (Rolph et al., 2006). In line with evidence obtained in Chapter 3, analysis of specific gene expression in relation to $CD8^+$ cDC reveals that L-DC resemble myeloid type DC.

Further investigation between L-DC and $CD8^-$ cDC revealed multiple genes specifically expressed by L-DC including *Clec4e*, *Pilrb2*, *Fabp4*, *Plaur*, *Dnahc12*, *Fgd4*, *Xdh* and *Smpdl3b*, which were also observed in the comparison of L-DC and $CD8^+$ cDC (Figures 6.9 and 6.10). In addition, L-DC specifically expressed *Ms4a4a*, *Zfyve9* and *Lilra5* over $CD8^-$ cDC (Figure 6.10). LILRA5 is expressed by macrophages and induces production of proinflammatory cytokines and IL-10 in a rheumatoid arthritis model (Mitchell et al., 2008). ZFYVE9 participates in TGF-β signalling by recruiting and interacting with SMAD2 and SMAD3 proteins (Tang et al., 2010). Lastly, MS4A4A is a novel protein which is part of a signalling complex. In summary, L-DC specifically express multiple genes involved in signalling pathways which are distinct from $CD8^-$ cDC.

Sorted $CD8^+$ cDC and $CD8^-$ cDC were also found to express many genes specific to each subset (Figure 6.11). $CD8^+$ cDC showed upregulation of *Clec9a*,

**Figure 6.10 Genes specifically expressed in either $CD8^-$ cDC or L-DC.**

ANOVA analysis was used to make pairwise comparisons of gene expression between the $CD8^-$ cDC and L-DC subset. Genes specifically expressed in either $CD8^-$ cDC or L-DC were selected based on the criteria of signal value in one subset $\leq 50$, and signal value in the other $\geq 150$. This gave a dataset of 71 genes (see Table A.5). Only those showing $\geq 8$ fold difference in signal value are shown.

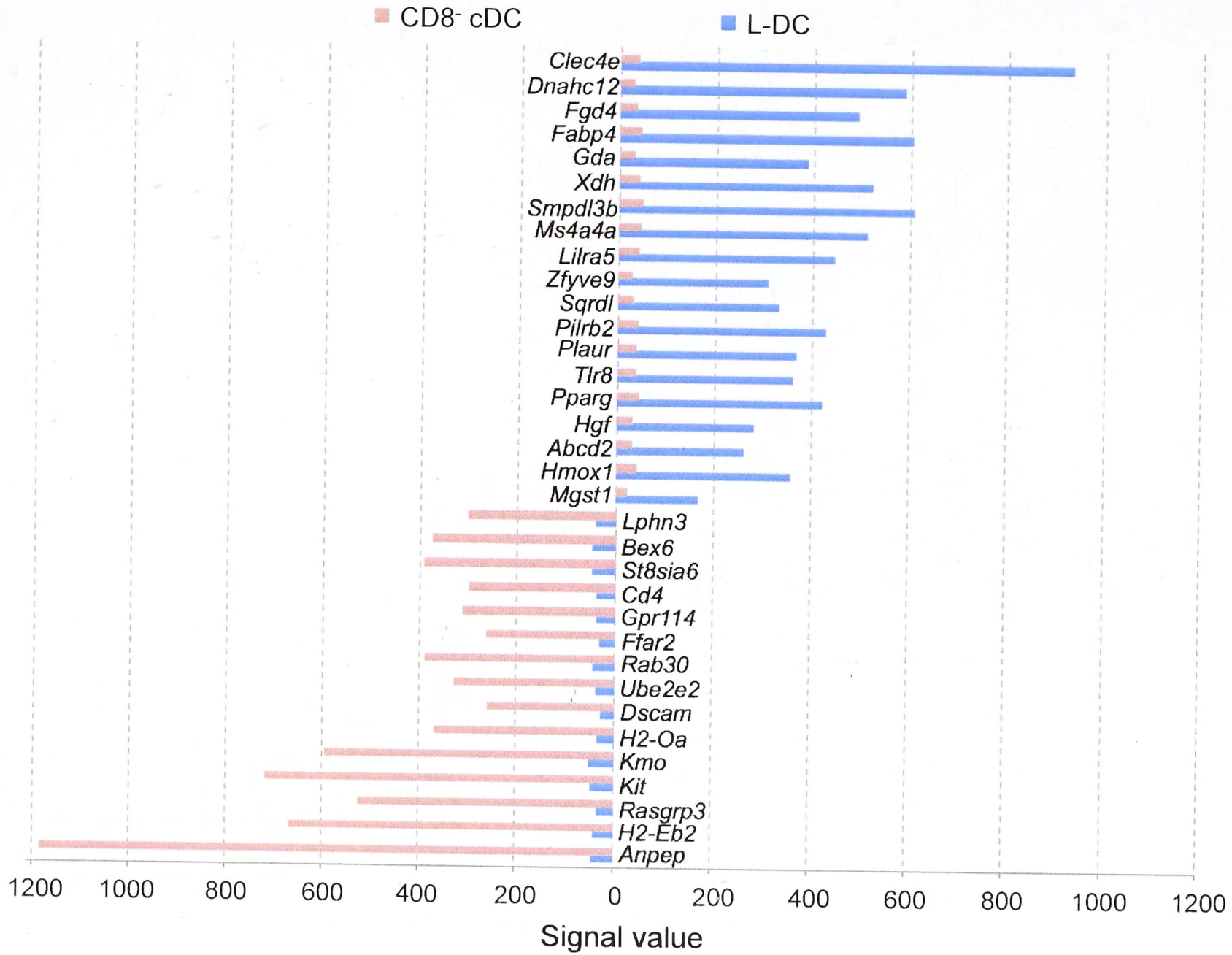
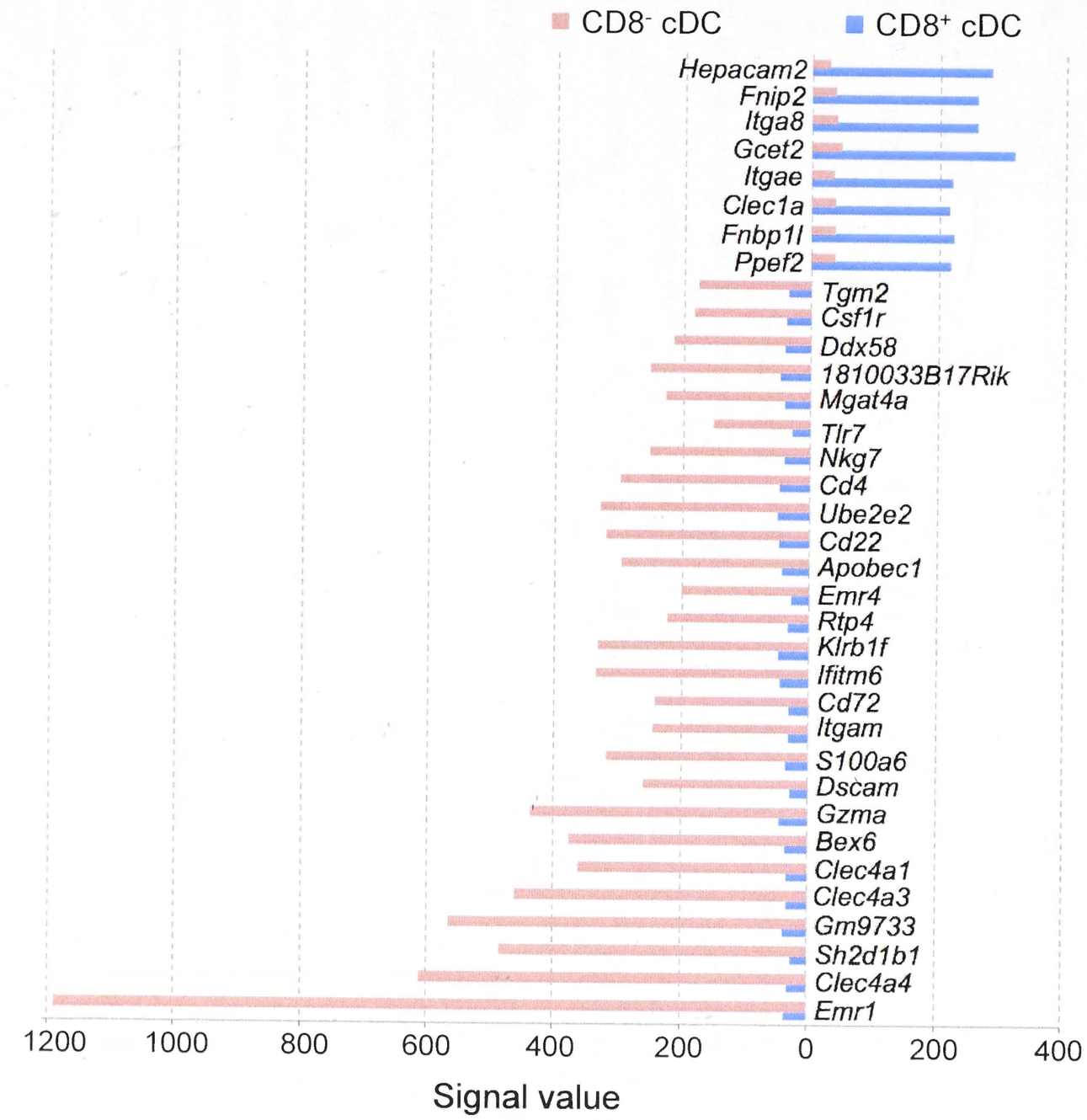

CD8- cDC
L-DC
Clec4e
Dnahc12
Fgd4
Fabp4
Gda
Xdh
Smpdl3b
Ms4a4a
Lilra5
Zfyve9
Sqrdl
Pilrb2
Plaur
Tlr8
Pparg
Hgf
Abcd2
Hmox1
Mgst1
Lphn3
Bex6
St8sia6
Cd4
Gpr114
Ffar2
Rab30
Ube2e2
Dscam
H2-Oa
Kmo
Kit
Rasgrp3
H2-Eb2
Anpep
1200
1000
800
600
400
200
0
200
400
600
800
1000
1200
Signal value

**Figure 6.11 Genes specifically expressed in either $CD8^+$ cDC or $CD8^-$ cDC.**

ANOVA analysis was used to make pairwise comparisons of gene expression between the $CD8^+$ cDC and $CD8^-$ cDC subsets. Genes specifically expressed in either $CD8^+$ cDC or $CD8^-$ cDC subsets were selected based on the criteria of signal value in one subset $\leq 50$, and signal value in the other $\geq 150$. This gave a dataset of 35 genes (shown).


CD8- cDC
CD8+ cDC
Hepacam2
Fnip2
Itga8
Gcet2
Itgae
Clec1a
Fnbp1l
Ppef2
Tgm2
Csf1r
Ddx58
1810033B17Rik
Mgat4a
Tlr7
Nkg7
Cd4
Ube2e2
Cd22
Apobec1
Emr4
Rtp4
Klrb1f
Ifitm6
Cd72
Itgam
S100a6
Dscam
Gzma
Bex6
Clec4a1
Clec4a3
Gm9733
Sh2d1b1
Clec4a4
Emr1
1200
1000
800
600
400
200
0
200
400
Signal value

*Cd8*, *Anpep*, *Tlr11* and *5430435g22Rik*, while $CD8^-$ cDC showed upregulation of *H2-Eb2*, *Cd4*, *Dscam* and *St8sia6* (Miller et al., 2012; Shortman and Heath, 2010) (Table A.1). In addition, pairwise comparison between $CD8^+$ cDC and $CD8^-$ cDC to identify specifically expressed genes revealed multiple known DC genes. $CD8^+$ cDC specifically expressed *Hepacam2* and *Itgae*, while $CD8^-$ cDC specifically expressed *Emr1*, *Emr4*, *Clec4a4a*, *Clec4a1*, *Clec4a3*, *Gm9733*, *Dscam*, *Itgam* and *Apobec1* (Figure 6.11). The above analyses confirm that the sorted cDC subsets were accurately identified and sorted in line with cDC subsets described in the literature.

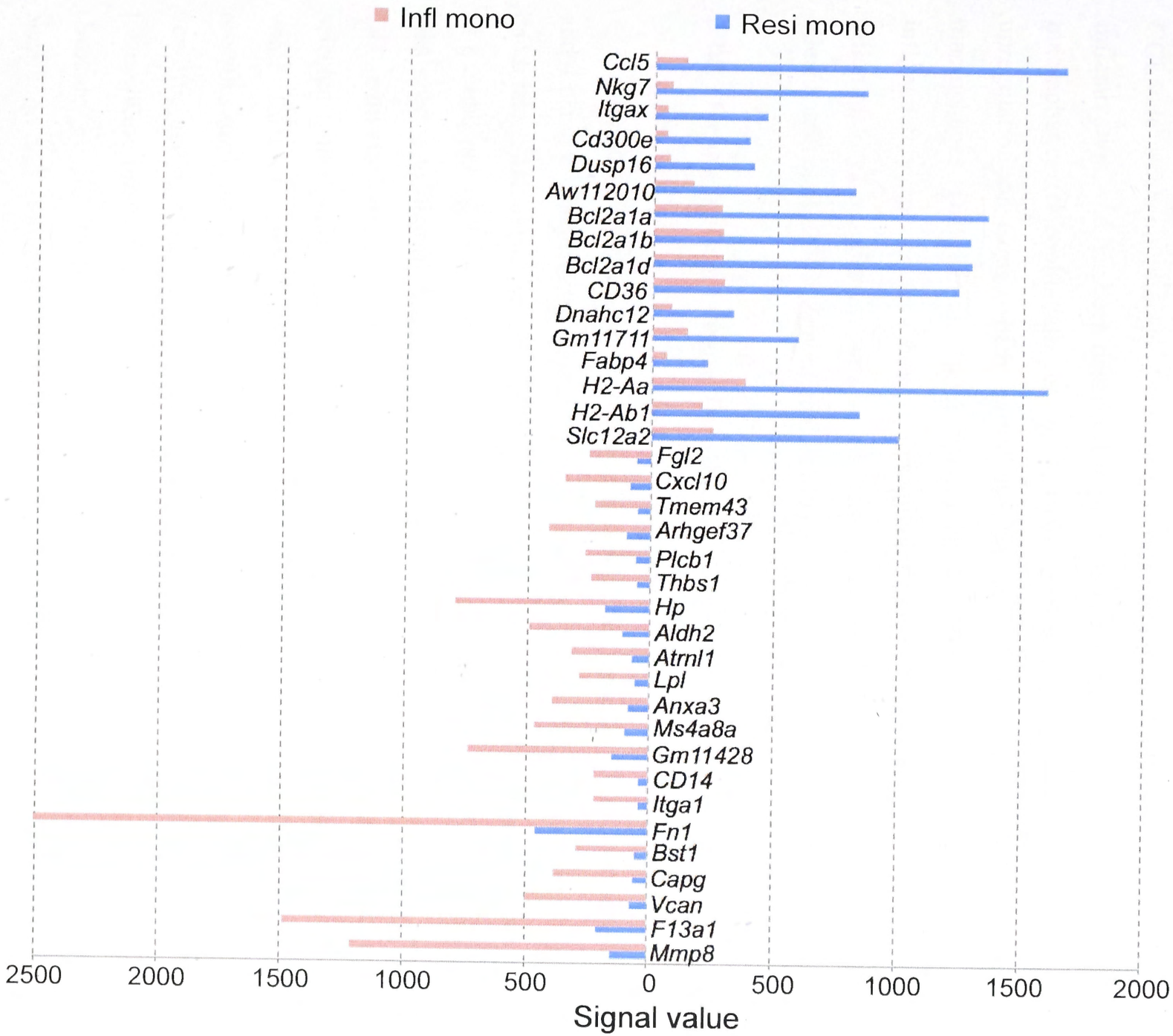
### 6.2.7 Identification of markers which distinguish resident and inflammatory monocyte subsets

Both resident monocytes and inflammatory monocytes have been shown here to be closely related in terms of origin and phenotype. Previously, blood-derived inflammatory monocytes were described as precursors of resident monocytes (Sunderkötter et al., 2004; Varol et al., 2007), although their relationship is still under discussion. To further distinguish these two monocyte subsets in spleen, genes specifically expressed by either resident or inflammatory monocytes were identified by pairwise comparison. However, only genes specific to resident monocytes could be identified, suggesting that inflammatory monocytes are closely related to resident monocytes in spleen in terms of function or lineage. Amongst the small number of genes identified as specifically expressed by resident monocytes, half are expressed by natural killer cells (data not shown). This suggests that sorted resident monocytes may be contaminated with a subset of natural killer cells. These genes were therefore not identified amongst genes upregulated in resident monocytes over inflammatory monocytes or L-DC. This was also seen in Chapter 3.

In a comparison of resident and inflammatory monocytes, resident monocytes upregulated genes encoding known markers like *Ccl5*, *Itgax*, *Cd300e*, *Dusp16*, *Cd36*, *H2-Ab1* and *Fabp4*, while inflammatory monocytes upregulated *Mmp8*, *F13a1*, *Vcan*, *Cd14* and *Fn1* (Figure 6.12). These findings were in line with the literature that compared gene expression profiles of murine blood monocyte subsets (Ingersoll et al., 2010). In line with the staining and gating strategy used, resident monocytes showed upregulation of *Itgax* also know as CD11c. Upregulation of *H2-Ab1* and *H2-*

**Figure 6.12 Genes upregulated in either resident monocytes or inflammatory monocytes.**

ANOVA analysis was used to make pairwise comparisons between gene expression in inflammatory and resident monocytes. Genes were selected which showed ≥ 4 fold change in signal value in either resident monocytes (Resi mono) or inflammatory monocytes (Infl mono) and signal value in the both subsets ≥ 50. This gave a dataset of 37 genes (shown).


Infl mono
Resi mono
Ccl5
Nkg7
Itgax
Cd300e
Dusp16
Aw112010
Bcl2a1a
Bcl2a1b
Bcl2a1d
CD36
Dnahc12
Gm11711
Fabp4
H2-Aa
H2-Ab1
Slc12a2
Fgl2
Cxcl10
Tmem43
Arhgef37
Plcb1
Thbs1
Hp
Aldh2
Atrnl1
Lpl
Anxa3
Ms4a8a
Gm11428
CD14
Itga1
Fn1
Bst1
Capg
Vcan
F13a1
Mmp8
2500
2000
1500
1000
500
0
500
1000
1500
2000
Signal value

*Aa* could indicate the potential for resident monocytes to express MHCII upon activation. DUSP16 is a dual-specificity phosphatase that can regulate mitogen-activated protein kinase involved in transduction of extracellular signals to the nucleus for gene transcription (Masuda et al., 2001). It has also been described to selectively regulate cytokine production by myeloid cells (Niedzielska et al., 2014). CCL5 is a chemokine involved in the recruitment of leukocytes to sites of inflammation, and has been described to promote recruitment and survival of human macrophages (Keophiphath et al., 2010). Inflammatory monocytes showed upregulation of *Capg* which participates in control of actin-based motility in macrophages (Dabiri et al., 1992; Pellieux et al., 2003). In line with the literature, inflammatory monocytes showed upregulation of CD14, a marker on human inflammatory monocytes which acts as a co-receptor for TLR4 signalling (Geissmann et al., 2003; Zanoni et al., 2011).

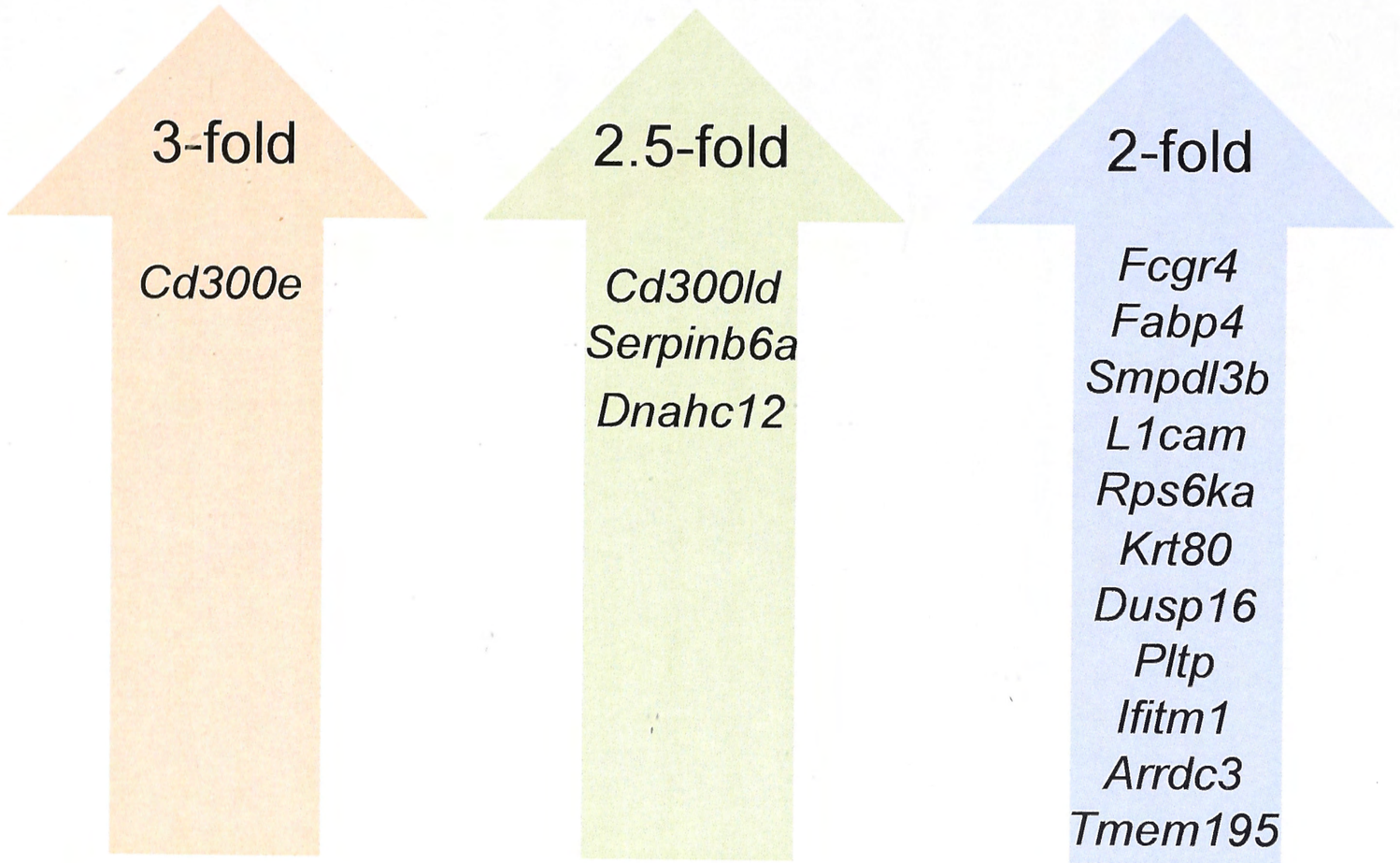
### 6.2.8 Gene expression unique to L-DC but not other subsets

Efforts were made to identify a delineating phenotype for L-DC in Chapters 3 and 4 using available antibodies. These markers have proven helpful in delineating a candidate L-DC subset separate from other dendritic and myeloid subsets. However, in the absence of a specific L-DC marker, more definitive gene expression analysis was needed to identify L-DC. Genes expressed by L-DC, but not any other sorted DC or myeloid subset prepared in this study, were therefore identified. Genes were selected with signal values $\geq 150$, and with a relative fold difference between signal values for L-DC and all other subsets of at least 2-fold (Figure 6.13). In line with previous analyses, L-DC specifically upregulated *Cd300e* at $\geq$ 3-fold over all other dendritic and myeloid subsets. CD300E is important for activation of monocytes and for cytokine production. CD300E-primed DC also induce stronger T cell activation (Brckalo et al., 2010). L-DC also showed specific upregulation of *Cd300ld*, *Serpinb6a* and *Dnahc12* at $\geq$ 2.5-fold levels (Figure 6.13). Genes upregulated $\geq$ 2-fold in L-DC over other subsets were mainly proteases, adhesion proteins and transmembrane proteins which are more commonly expressed genes. In summary, markers discovered here which may delineate L-DC amongst other splenic DC and myeloid subsets include CD300E, CD300LD, CD9 and SERPINB6A.

**Figure 6.13 Genes specifically expressed by L-DC and not other dendritic and myeloid subsets.**

Genes were selected based on the criteria of signal value in L-DC ≥ 150, with fold difference between L-DC and the lowest expressing subset ≥ 2 fold. Genes showing ≥ 2.0 fold, ≥ 2.5 fold and ≥ 3 fold upregulation in L-DC are listed.

# Markers Unique to L-DC



## 6.3 Discussion

Gene profiling studies have been used to identify the L-DC lineage and to identify new markers which distinguish the L-DC subset from other splenic dendritic and myeloid subsets. Potential new markers of interest include CD300E, CD300LD and CD9. While CD9 is more commonly expressed by leukocytes and endothelial cells (Barreiro et al., 2008), CD300E and CD300LD have more restricted expression by DC subsets (Gasiorowski et al., 2013). Unfortunately, the only antibodies available for CD300E are specific for human and do not crossreact with murine molecules (data not shown). The studies described here also identify a close relationship in terms of gene expression between L-DC and the $CD11c^{lo}$ resident monocyte subset in spleen. The resident monocyte population is quite distinct from inflammatory monocytes in spleen despite evidence for common function. Both $CD8^+$ cDC and $CD8^-$ cDC gene profiles were found to be consistent with the literature indicating that the staining and sorting protocols established in Chapter 4 are accurate for delineation of these subsets. Lastly, the gene profile of eosinophils was quite distinct from the other dendritic and myeloid subsets isolated, suggesting a distinct lineage origin for these cells. This result is consistent with evidence that the eosinophil develops from a granulocyte/macrophage progenitor instead of macrophage/dendritic cell progenitors (Iwasaki and Akashi, 2007; McNagny and Graf, 2002; Weissman et al., 2001).

In terms of overall gene expression of isolated splenic subsets, PCA analysis indicated a close relationship between L-DC, resident monocytes and inflammatory monocytes. Both $CD8^+$ cDC and $CD8^-$ cDC were more closely associated. Despite their common origin from the CDP, $CD8^+$ cDC and $CD8^-$ cDC develop quite distinct functions (Figure 6.2). Gene expression between eosinophils and L-DC, and eosinophils and inflammatory monocytes, showed the largest variation, while L-DC and resident monocytes, and L-DC and inflammatory monocytes showed the least variation in gene expression. Hierarchical clustering identified L-DC as closely related to resident monocytes, and $CD8^+$ cDC and $CD8^-$ cDC subsets were also closely related. The close relationship between gene profiles for L-DC and resident monocytes raises questions about a possible common lineage origin. It is yet to be

determined whether one is a precursor of the other, or whether they reflect functionally distinct progeny of a common progenitor endogenous to spleen.

Gene expression profiles obtained for resident monocytes compared with inflammatory monocytes were consistent with gene expression described in the literature for monocytes. Both monocyte subsets expressed *Csf1r* and *Ccr2* which encode receptors essential for monocyte development and migration, respectively (Figure 6.4 A-D) (Auffray et al., 2009; Geissmann et al., 2008). Resident monocytes did not show specific gene expression distinct from inflammatory monocytes, although a number of genes were upregulated in expression. Resident monocytes upregulated expression of *Ccl5, Dusp16, Cd36, Fabp4* and *H2-Ab1*, while inflammatory monocytes upregulated *Fn1, F13a1*, *Mmp8*, *Vcan* and *Cd14* expression (Figure 6.12). The gene expression profiles of both resident and inflammatory monocytes were very similar, making it difficult to identify markers specific for each subset. Similarity in gene profile could be attributed to their development and a common lineage origin, or a common progenitor (Fogg et al., 2006). For example, previously it was reported that Ly6C$^{hi}$ inflammatory monocytes are a precursor of Ly6C$^{lo}$ resident monocytes, although that study involved peripheral blood rather than splenic monocytes (Sunderkötter et al., 2004).

When gene profiles of L-DC were compared with those of the cDC subsets, L-DC was shown to upregulate genes encoding both dendritic and myeloid cell markers like *Clec4a4*, *Emr4*, *Zeb2*, *Krt80*, *Itgam* and *Csfr1* (Caminschi et al., 2001a; Dudziak et al., 2007; Idoyaga et al., 2013; Qi et al., 2012; Windoffer et al., 2011) (Table 6.1). When the L-DC gene profile was compared with that of the two monocyte subsets, it was found that L-DC upregulated *Cd300e* and *Cd9* (Table 6.2-6.3). Both CD300E and CD9 regulate the function of DC, monocytes, mo-DC and T cells (Aguilar et al., 2004; Borrego, 2013; Brckalo et al., 2010). These results are consistent with Chapter 3 findings in that L-DC were found to express multiple markers that regulate T cell function. In addition to CD300E and CD9, data mining to identify new markers for L-DC revealed CD300LD, SERPINB6A and DNAHC12 as further markers of interest (Figure 6.13). CD300LD belongs to the same family as CD300E, and participates in signal transduction and production of pro-inflammatory cytokines (Comas-Casellas et al., 2012; Gasiorowski et al., 2013). *Serpinb6a*

encodes a protein essential for protection against cytotoxic granules (Zhang et al., 2006), while *Dnahc12* encodes a protein that forms part of dynein (Roberts et al., 2013).

Overall, the L-DC subset of $CD11b^{hi}CD11c^{lo}Ly6C^{-}Ly6G^{-}CD43^{+}Siglec\text{-}F^{-}$ cells has a gene profile showing a close lineage relationship with that of resident monocytes. However, L-DC express many genes in common to cells of the dendritic lineage, which are not expressed by resident monocytes. Overall, L-DC represent a novel cell type with a gene expression profile resembling both the cDC and monocyte lineages. To further analyse the relationship between L-DC, and other dendritic and myeloid subsets in spleen, mutant mouse models with a mutation in genes encoding growth factors, cytokines or receptors were investigated to determine how specific mutation affected the development of each of the subsets described here.

Chapter 7

# L-DC development is distinct from splenic dendritic and myeloid cell subsets

## 7.1 Introduction

In the steady-state, spleen contains multiple subsets of dendritic cells (DC) and myeloid cells. The average half-life of conventional (c) DC is about three days, while monocytes have a half-life of 1 day (Kamath et al., 2002; van Furth and Cohn, 1968). Thus, splenic cDC and monocytes need to be constantly replenished from precursors. Hematopoiesis occurs within bone marrow where hematopoietic stem cells (HSC) give rise to multipotent progenitors (MPP), which in turn generate common lymphoid progenitors (CLP) and common myeloid progenitors (CMP). CMP give rise to the macrophage/dendritic cell progenitors (MDP) (Fogg et al., 2006) which generate common dendritic progenitors (CDP) (Liu et al., 2009; Onai et al., 2007). MDP generate cDC and monocytes, which develop to become macrophages in tissues, whilst CDP exclusively generate cDC and pDC (Liu et al., 2009). Monocytes and plasmacytoid (p) DC exit bone marrow as mature cells and pre-pDC, but cDC leave bone marrow as pre-cDC that further differentiate and divide in spleen (Naik et al., 2006; Naik et al., 2007). The lineages of cDC and myeloid cells have been extensively studied, but many questions remain to be answered.

Our understanding of L-DC development is still incomplete, although L-DC are thought to originate from HSC or hematopoietic progenitor cells (HPC) that seed spleen during embryogenesis (Petvises and O'Neill, 2014). Other examples of tissue-specific DC development have been reported including Langerhans cells, which derive from a local pool of self-renewing HPC in the epidermis (Merad et al., 2004; Merad et al., 2002). Recent studies on neonatal spleen showed that L-DC could be detected as early as embryonic day (E) 18.5, while cDC were detected later at 4 days after birth (Petvises and O'Neill, 2014). This suggests that L-DC are distinct from the cDC lineage, and could develop independently of hematopoiesis in bone marrow. In addition, culturing sorted CDP or MDP over supporting stromal cells failed to induce L-DC production (Petvises and O'Neill, 2014). These findings suggest that L-DC are distinct from both the cDC and monocyte lineage. Recent studies have described a lineage of macrophages that develops independently of hematopoiesis in bone marrow, arising from yolk-sac HSC or HPC (Schulz et al., 2012). These cells are phenotypically distinct from bone marrow-derived macrophages, although their

functional capacity still remains to be investigated (Schulz et al., 2012). In addition, the relative contribution of yolk sac-derived macrophages verses bone marrow-derived macrophages to tissue sites still remains to be investigated fully. Recent studies have however confirmed that *c-Myb* is essential in the development of bone marrow-derived but not yolk sac-derived myeloid cells, and in *c-Myb* mutant mice, these subset changes can be identified (Schulz et al., 2012).

Here the development of L-DC has been investigated in a number of mutant mouse models known to impact the development of DC and myeloid subsets. The GM-CSF and FLT3 ligand (FLT3L) growth factors are known to be important in the differentiation of DC subsets (Xu et al., 2007). In order to investigate L-DC development in relation to cDC, splenic DC and myeloid subsets were investigated in *GM-CSF*$^{-/-}$ and *Flt3L*$^{-/-}$ mutant mice. The BATF3 transcription factor was recently described as essential for CD8$^{+}$ cDC development (Jaiswal et al., 2013; Tussiwand et al., 2012), and the importance of this factor in L-DC development has therefore been investigated by analysis of the DC subset composition of *Batf-3*$^{-/-}$ mouse spleen.

One consideration in L-DC development is whether these cells which are found only in spleen develop in response to inflammatory signals. Such a model may preclude their development in the steady-state. In terms of their ability to respond to environmental signals, both dendritic and myeloid cells express Toll like receptors (TLR) on their cell surface that recognise pathogen-associated molecule patterns (PAMP) which activate innate immunity. Signalling through a range of TLR molecules is mediated by either MYD88 or TRIF (Takeda et al., 2003; West et al., 2006). To investigate the role of TLR signalling in L-DC development, *MyD88*$^{-/-}$ or *Trif*$^{-/-}$ mice have been analysed for the presence of DC subsets in spleen. Studies on L-DC in this chapter, also encompass an analysis of resident monocytes, since this subset has been found in Chapters 4 and 6 to resemble L-DC in terms of their phenotype and gene expression.

## 7.2 Results

### 7.2.1 L-DC development occurs independently of growth factors which regulate cDC development

Fms-like tyrosine kinase 3 (FLT3) signalling has been described as essential for cDC development in the spleen in the steady-state (McKenna et al., 2000). Adminstration of FLT3L during *in vitro* and *in vivo* cell development leads to an increase in the number of splenic cDC and pDC (Maraskovsky et al., 1996; Pulendran et al., 1997; Shurin et al., 1997). Similarly, knockdown of *Flt3L* in mice leads to a two- to three- fold drop in the numbers of splenic cDC and pDC in adult mice (McKenna et al., 2000). To investigate if L-DC development is equally dependent on FLT3L, the representation of cDC and L-DC was analysed relative to DC and myeloid subsets in adult wild type and *Flt3L*$^{-/-}$ mice. Myeloid cells were assessed as control subsets.

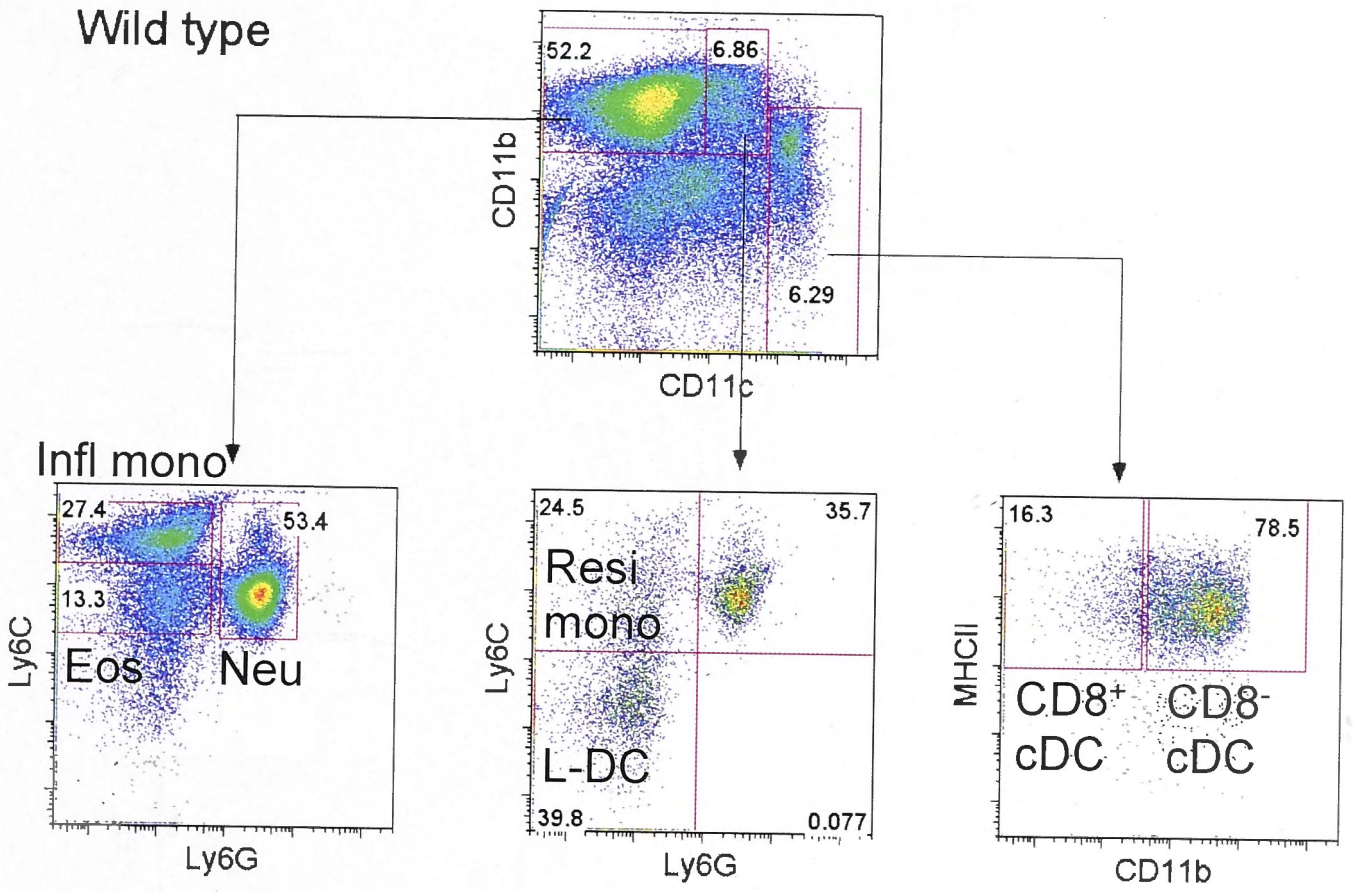
$CD8^+$ cDC were gated as $CD11b^-CD11c^{hi}MHCII^+$ cells, while $CD8^-$ cDC were gated as $CD11b^+CD11c^{hi}MHCII^+$ cells (Figure 7.1 A). In line with the literature (Beaudin et al., 2014; Karsunky et al., 2003), a six-fold drop in percent $CD11c^{hi}$ cells was observed in *Flt3L*$^{-/-}$ mice compared with wild type mice. However, the relative proportion of $CD8^+$ cDC and $CD8^-$ cDC was unaffected (Figure 7.1 A). A significant 5.5-fold drop in percent $CD8^-$ cDC amongst total dendritic and myeloid cells, and a significant 2-fold drop in percentage of $CD8^+$ cDC were observed in multiple *Flt3L*$^{-/-}$ mice compared with wild type mice (Figure 7.1 B). There was also a significant drop in the percentages of inflammatory and resident monocytes in *Flt3L*$^{-/-}$ mice (Figure 7.1 C). In contrast, neither L-DC nor eosinophils were affected by loss of *Flt3L* expression. Lastly, the percentage of neutrophils increased in *Flt3L*$^{-/-}$ mice. These results predict a role for FLT3L in the development of cDC, inflammatory and resident monocytes, but not L-DC and eosinophils. The increase in neutrophils could reflect inflammation in a mouse model with reduced immune capacity.

During inflammation, inflammatory monocytes can give rise to DC that produce TNF-α and inducible NO synthase. These are called Tip-DC (Auffray et al., 2009; Geissmann et al., 2003; Serbina et al., 2008). Granulocyte-macrophage

**Figure 7.1 L-DC development occurs independently of Flt3 ligand.**

Splenocytes were harvested from C57BL/6J-*Flt3L*$^{tm1Imx}$ (Flt3-ligand KO) and C57BL/6J (wild type) mice, and red blood cells lysed prior to T and B cell depletion. Cells were stained with antibodies specific for CD11b (M1/70, Pe-Cy7), CD11c (N418, APC), MHC-II (M5/114.15.2, Pacific Blue), Ly6C (Al-21, FITC) and Ly6G (1A8, PE). Prior to analysis, cells were stained with propidium iodide (PI, 1μg/ml) for gating live (PI$^-$) cells. A) Gating strategy to distinguish myeloid and DC subsets. Myeloid cells were initially gated as CD11b$^{hi}$CD11c$^-$ cells, and further divided to give inflammatory monocytes (Infl mono: Ly6C$^{hi}$Ly6G$^-$ cells), eosinophils (Eos: Ly6C$^+$Ly6G$^-$ cells) and neutrophils (Neu: Ly6C$^+$Ly6G$^+$ cells). L-DC were gated as CD11b$^{hi}$CD11c$^{lo}$Ly6C$^-$ Ly6G$^-$ cells, while resident monocytes were gated as CD11b$^{hi}$CD11c$^{lo}$Ly6C$^+$Ly6G$^-$ cells. Conventional dendritic cells (cDC) were gated as CD11c$^{hi}$CD11b$^{+/-}$ cells and further delineated as CD8$^+$ cDC (MHCII$^+$CD11b$^-$) and CD8$^-$ cDC (MHCII$^+$CD11b$^+$) subsets. Gates were set based on fluorescence minus one controls, and numbers in gates represent % specific binding. Individual mice were analysed. B) Percent cDC subsets are shown relative to the total dendritic and myeloid cell population. C) Percent myeloid cells and L-DC are shown relative to the total dendritic and myeloid cell population. A bar is used to show mean values. Red boxes indicate a significant change relative to wild type using Student's *t*-test (p value $\leq 0.01$).

A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CD11c
Infl mono
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Eos
Neu
Ly6C
Ly6G
24.5
35.7
Resi
mono
L-DC
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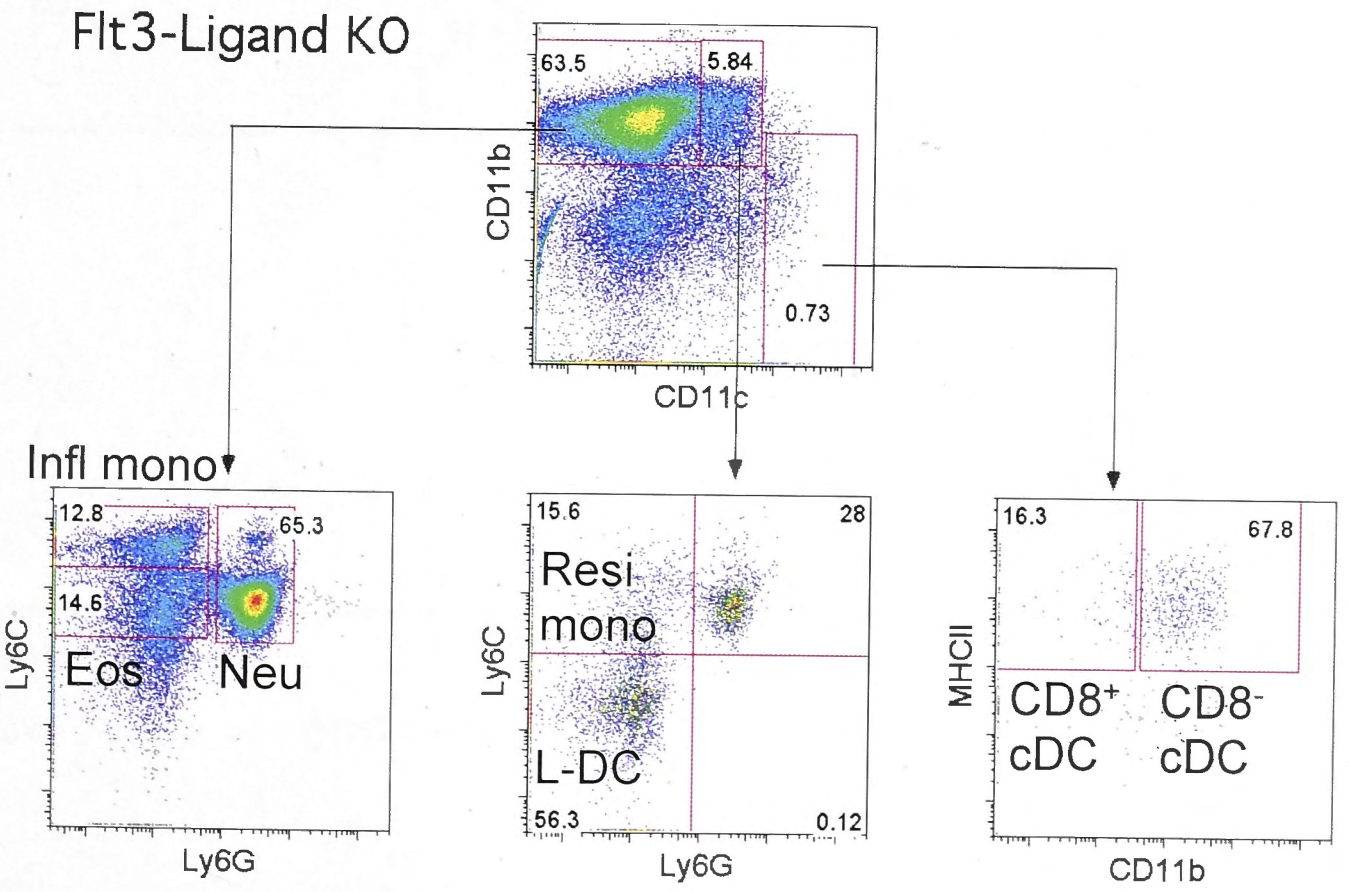
CD8+
cDC
CD8-
cDC
MHCII
CD11b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CD11b
CD11c
Infl mono
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65.3
14.6
Eos
Neu
Ly6C
Ly6G
15.6
28
Resi
mono
L-DC
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0.12
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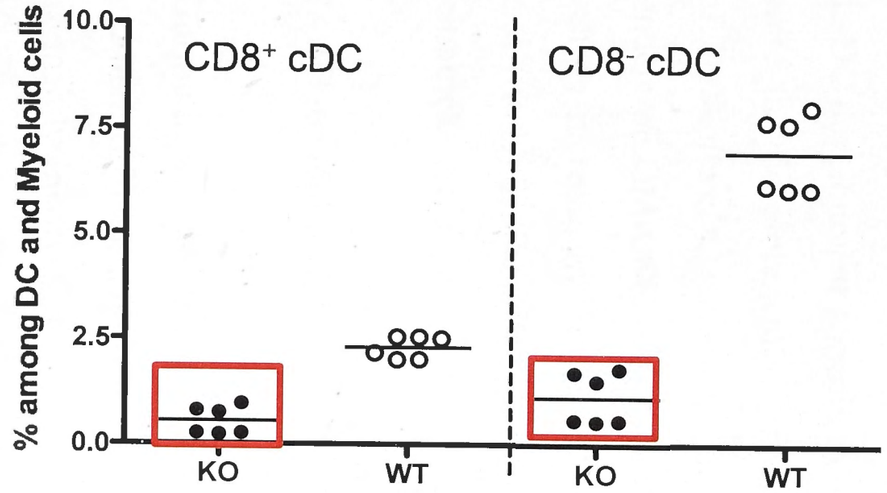
67.8
CD8+
cDC
CD8-
cDC
MHCII
CD11b

B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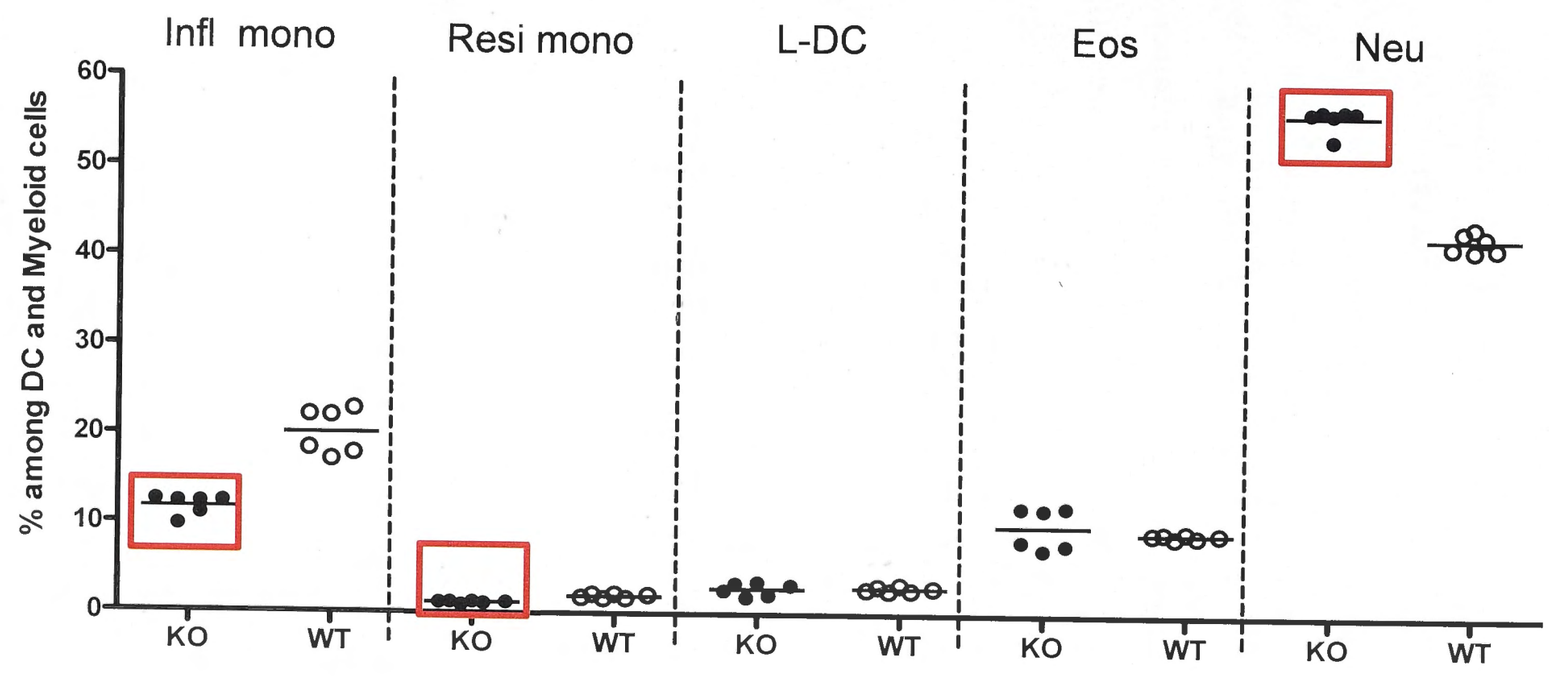
5.0
2.5
0.0
CD8+ cDC
CD8- cDC
KO
WT
KO
WT

C


% among DC and Myeloid cells
60
50
40
30
20
10
0
Infl mono
Resi mono
L-DC
Eos
Neu
KO
WT
KO
WT
KO
WT
KO
WT
KO
WT

colony-stimulating factor (GM-CSF) has been shown to induce the formation of inflammatory type DC after addition to cultures of BM progenitors (Berthier et al., 2000; Inaba et al., 1993; Xu et al., 2007). In order to investigate a role for GM-CSF in the development of splenic dendritic and myeloid subsets, changes in the population representation of cDC, L-DC and myeloid subsets was investigated in *GM-CSF*$^{-/-}$ mice. Previous studies have reported no reduction in the proportion of pDC and cDC in *GM-CSF*$^{-/-}$ mice (Kingston et al., 2009; Vremec et al., 1997). In this study however, both $CD8^{+}$ cDC and $CD8^{-}$ cDC were found to be present in significantly higher percentage in *GM-CSF*$^{-/-}$ mice (Figure 7.2 B), while both resident and inflammatory monocytes demonstrated a significant drop in percent in *GM-CSF*$^{-/-}$ mice (Figure 7.2 C). The percentage of eosinophils in *GM-CSF*$^{-/-}$ mice was significantly increased, while the percentage of L-DC and neutrophils was unaffected.

L-DC development appears to occur independently of both FLT3L and GM-CSF, while the development of monocyte subsets is dependent on both factors (Figure 7.2). While cDC subsets are dependent on FLT3L for development, they are not dependent on GM-CSF. In fact, in the absence of GM-CSF, there appears to be a compensatory increase in the development of cDC as well as eosinophils (Figure 7.2). These results distinguish L-DC from both cDC and myeloid subsets, in that L-DC development occurs independently of both FLT3L and GM-CSF.

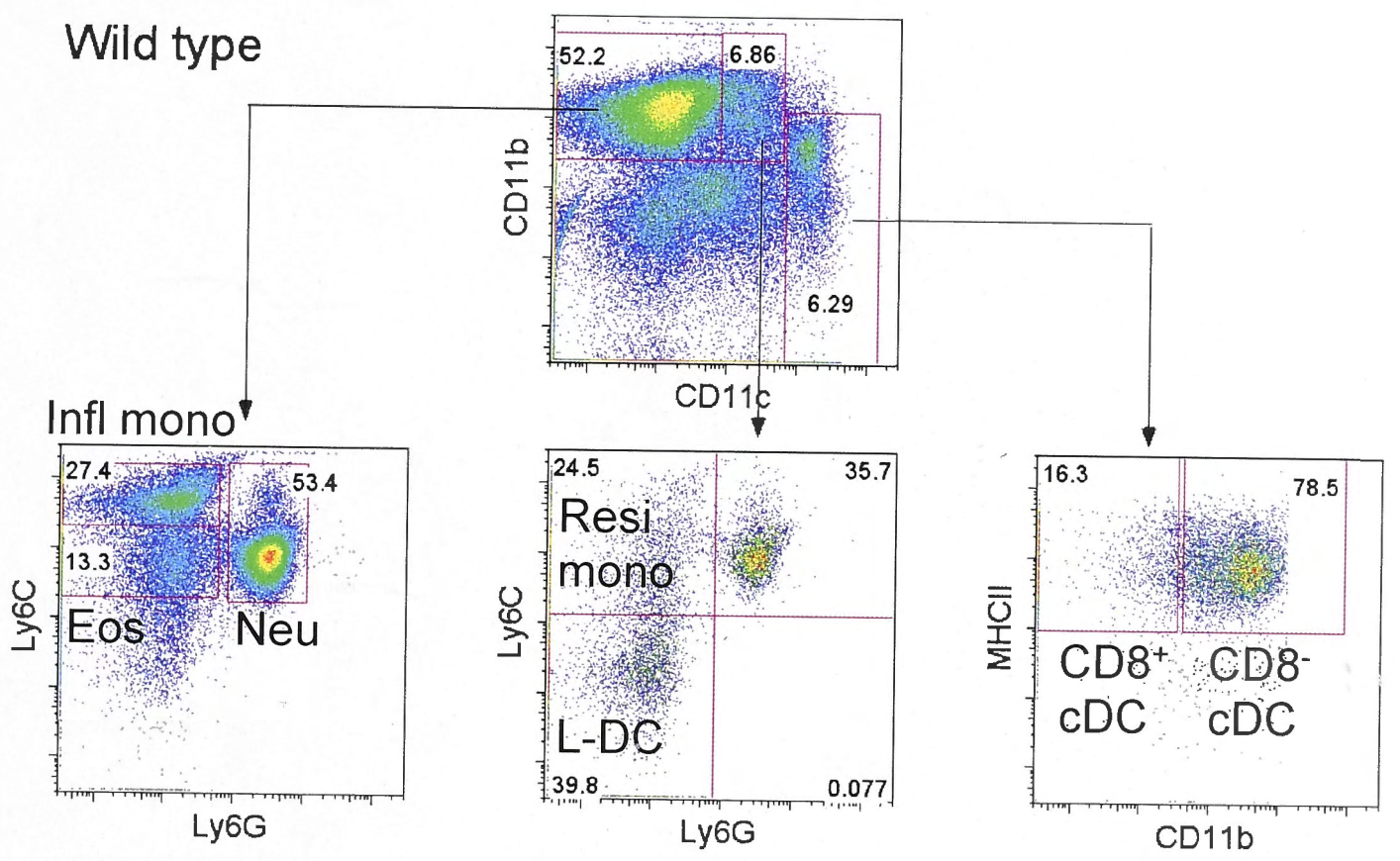
### 7.2.2 L-DC development occurs independently of inflammatory signals

During inflammation, the engagement of pathogen molecules to TLR on HSC can trigger their differentiation (Boiko and Borghesi, 2012; Nagai et al., 2006). TLR signalling mainly involves two adaptor proteins: MYD88 and TRIF (Takeda et al., 2003; West et al., 2006). MYD88 is required for all signalling through all TLR except TLR3 (Takeda et al., 2003; West et al., 2006). TLR3 uses TRIF for signal transduction, while TLR4 uses MYD88 or TRIF in association with Toll-interleukin 1 receptor domain-containing adapter protein (TIRAP) or Trif-related adaptor molecule (TRAM), respectively (Takeda et al., 2003; West et al., 2006).

**Figure 7.2 L-DC development occurs independently of GM-CSF.**

Splenocytes were harvested from C57BL/6J-*Csf2*$^{tm1Ard}$ (GM-CSF KO) and C57BL/6J (wild type) mice, and red blood cells lysed prior to T and B cell depletion. A) Cells were stained and gated as described in the legend to Figure 7.1. Gates were set based on fluorescence minus one controls, and numbers in gates represent % specific binding. B) Percent cDC subsets are shown relative to the total dendritic and myeloid cell population. C) Percent myeloid cells and L-DC are shown relative to the total dendritic and myeloid cell population. A bar is used to show mean values. Red boxes indicate a significant change relative to wild type using Student's *t*-test (p value $\leq 0.01$).

A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6.86
6.29
CD11b
CD11c
Infl mono
27.4
53.4
13.3
Eos
Neu
Ly6C
Ly6G
24.5
35.7
Resi
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L-DC
39.8
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78.5
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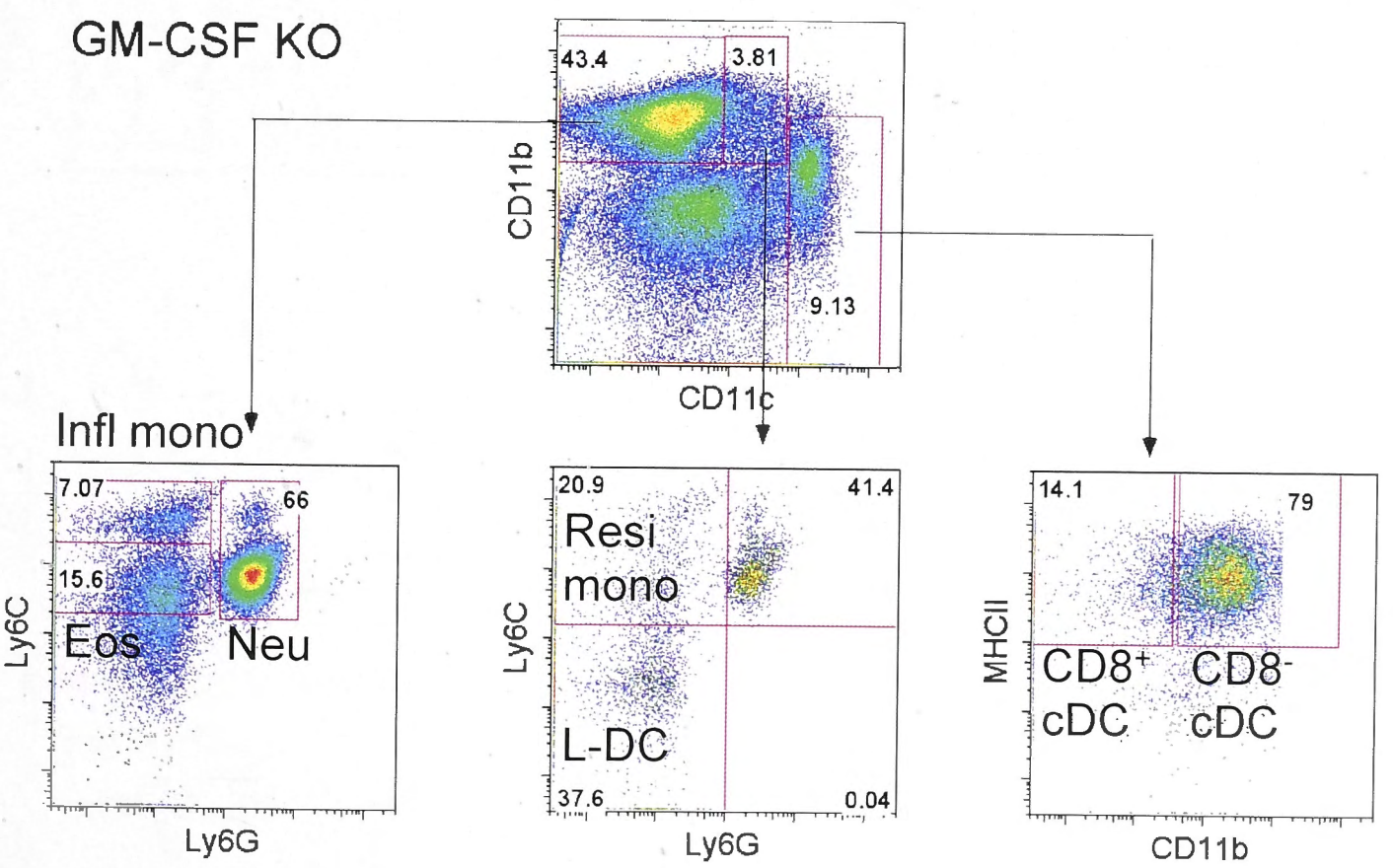
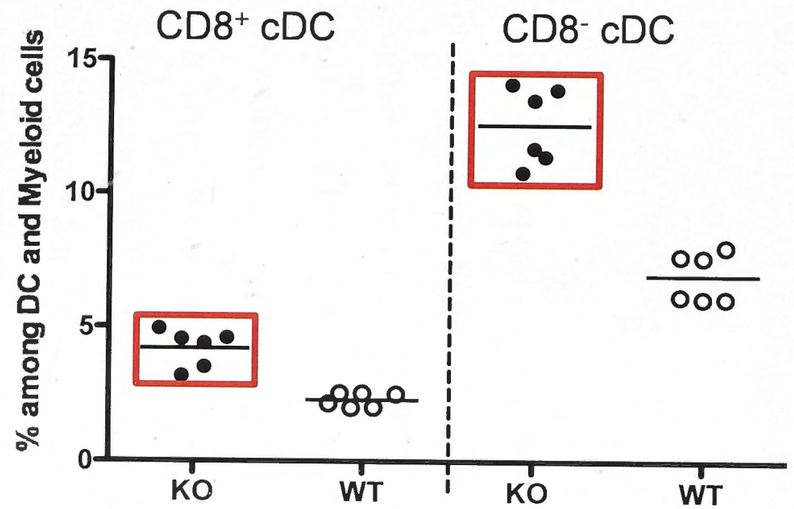
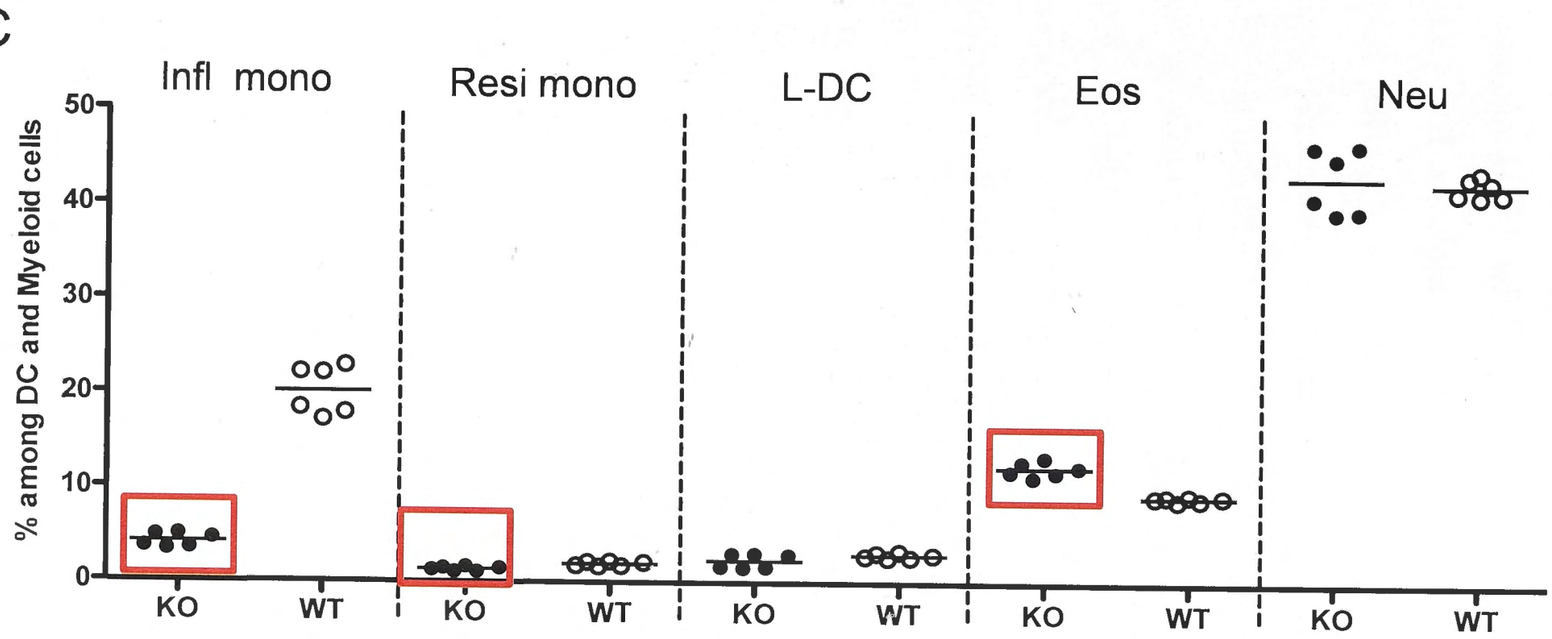
cDC
CD8-
cDC
MHCII
CD11b


GM-CSF KO
43.4
3.81
9.13
CD11b
CD11c
Infl mono
7.07
66
15.6
Eos
Neu
Ly6C
Ly6G
20.9
41.4
Resi
mono
L-DC
37.6
0.04
14.1
79
CD8+
cDC
CD8-
cDC
MHCII
CD11b

B


CD8+ cDC
CD8- cDC
% among DC and Myeloid cells
15
10
5
0
KO
WT
KO
WT

C


Infl mono
Resi mono
L-DC
Eos
Neu
% among DC and Myeloid cells
50
40
30
20
10
0
KO
WT
KO
WT
KO
WT
KO
WT
KO
WT

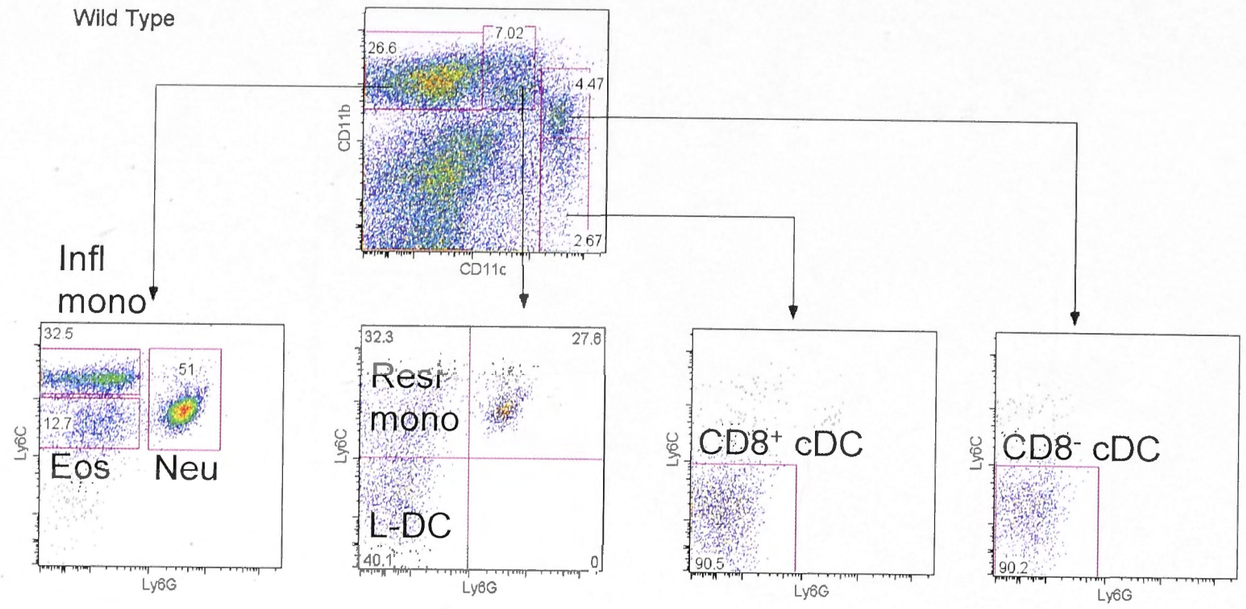
Analysis of L-DC development in *MyD88*$^{-/-}$ and *Trif*$^{/-}$ mice therefore represents a test of whether inflammatory signals are essential for the development of L-DC from HSC. To determine if TLR signalling plays a role in L-DC development, the percentage of L-DC amongst total dendritic and myeloid cells was analysed in *MyD88*$^{-/-}$, *Trif*$^{/-}$ and *MyD88*$^{-/-}$/*Trif*$^{/-}$ mice compared with wild type control mice. In addition, the representation of splenic DC and myeloid subsets was analysed concurrently to determine if knockout of TLR affects their development. In *MyD88*$^{-/-}$ mice, a significant 2.5-fold increase in the percentage of CD8$^{-}$ cDC was seen compared with wild type mice (Figure 7.3 B). On the other hand, eosinophils showed a significant, but small decrease in percentage (Figure 7.3 C). The populations of inflammatory monocytes, resident monocytes, L-DC and neutrophils were not significantly different in *MyD88*$^{-/-}$ mice compared with wild type mice (Figure 7.3 C). These results emphasise how development of L-DC and most other subsets occurs independently of inflammatory signals, with the exception of eosinophils. The increased number of CD8$^{-}$ cDC in mutant mice could be related to the presence of inflammatory or Tip DC amongst the CD8$^{-}$ cDC subset since these cells have a similar phenotype to CD8$^{-}$ cDC. The development of TipDC is likely to be dependent on TLR signalling.

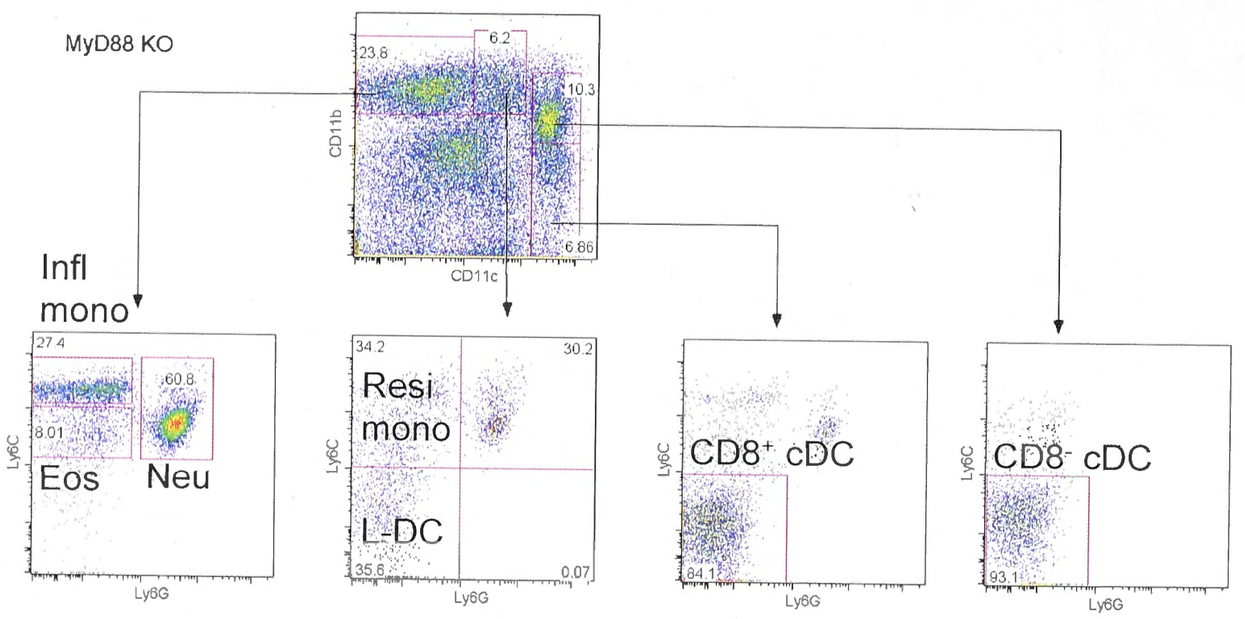
TLR3 is involved in recognition of double stranded RNA associated with viral infection (Alexopoulou et al., 2001). To investigate a possible role for TLR3 in the development of murine L-DC and cDC, the requirement for the TLR3 adaptor protein TRIF was investigated using *Trif*$^{/-}$ mice. Consistent with the literature, a significant reduction in the percentage of both CD8$^{+}$ cDC and CD8$^{-}$ cDC was observed in *Trif*$^{/-}$ mice compared with wild type mice, and associated with TLR3 signalling (Figure 7.4 B). Amongst the myeloid subsets, only resident monocytes showed a significant but small decrease in numbers compared with wild type mice, while neutrophils demonstrated a significant increase. The percentage of L-DC remained constant in *Trif*$^{/-}$ mice compared with wild type mice (Figure 7.4 C). These data reveal dependency for TLR3 signalling for development of cDC and resident monocytes. The increase in neutrophils could reflect the higher infection status of these mutant mice, or a compensatory effect.

**Figure 7.3 L-DC development occurs independently of MyD88 signalling.**

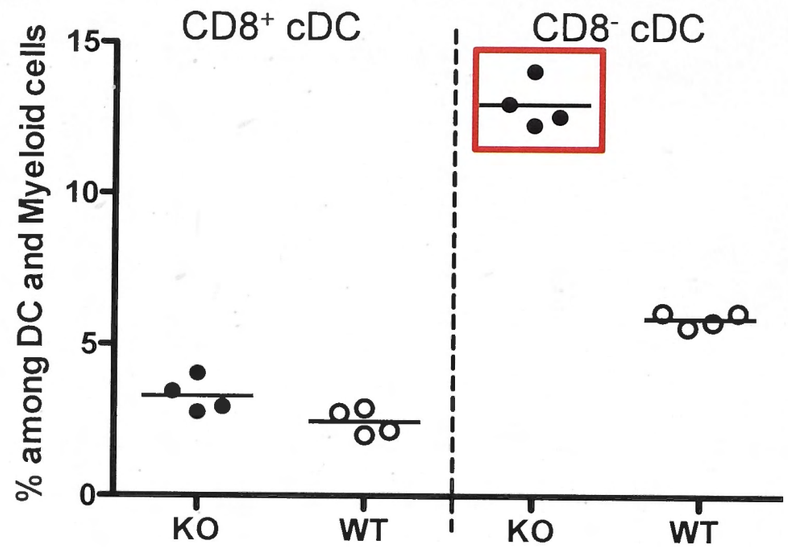
Splenocytes were harvested from C57BL/6J *MyD88*$^{-/-}$ (MyD88 KO) and C57BL/6J (wild type) mice, and red blood cells lysed prior to T and B cell depletion. Cells were stained with 2 distinct antibody cocktails. The first cocktail included antibodies to CD11b (M1/70, Pe-Cy7), CD11c (N418, APC), Ly6C (Al-21, FITC) and Ly6G (1A8, PE), while the second contained CD11b (M1/70, Pe-Cy7), CD11c (N418, APC), MHCII (AF6-120.1, FITC) and CD8 (53-6.7, PE). Prior to analysis, cells were stained with propidium iodide (PI, 1μg/ml) for gating live ($PI^-$) cells. A) Gating strategy to distinguish dendritic and myeloid subsets. Myeloid cells were initially gated as $CD11b^{hi}CD11c^-$ cells and further delineated into inflammatory monocytes (Infl mono: $Ly6C^{hi}Ly6G^-$ cells), eosinophils (Eos: $Ly6C^+Ly6G^-$ cells) and neutrophils (Neu: $Ly6C^+Ly6G^+$ cells). L-DC were gated as $CD11b^{hi}CD11c^{lo}Ly6C^-Ly6G^-$ cells, while resident monocytes were gated as $CD11b^{hi}CD11c^{lo}Ly6C^+Ly6G^-$ cells. $CD8^+$ cDC were gated as $CD11b^-CD11c^{hi}MHCII^+CD8^+$ cells, while $CD8^-$ cDC were gated as $CD11b^+CD11c^{hi}MHCII^+CD8^-$ cells. Gates were set based on fluorescence minus one controls, and numbers in gates represent % specific binding. Individual mice were analysed. A bar is used to show mean values. B) Percent cDC is shown relative to the total dendritic and myeloid cell population. C) Percent myeloid subsets and L-DC shown relative to the total dendritic and myeloid cell population. Red boxes indicate a significant change relative to wild type using Student's *t*-test (p value $\leq 0.01$). Blue boxes indicate a significant change relative to wild type using Student's *t*-test (p value $\leq 0.05$).

A


Wild Type
26.6
7.02
4.47
2.67
CD11b
CD11c
Infl mono
32.5
51
12.7
Ly6C
Eos
Neu
Ly6G
32.3
27.6
Resi mono
L-DC
40.1
0
CD8+ cDC
90.5
CD8- cDC
90.2


MyD88 KO
23.8
6.2
10.3
6.86
CD11b
CD11c
Infl mono
27.4
60.8
8.01
Ly6C
Eos
Neu
Ly6G
34.2
30.2
Resi mono
L-DC
35.6
0.07
CD8+ cDC
84.1
CD8- cDC
93.1

B


CD8+ cDC
CD8- cDC
% among DC and Myeloid cells
15
10
5
0
KO
WT
KO
WT

C

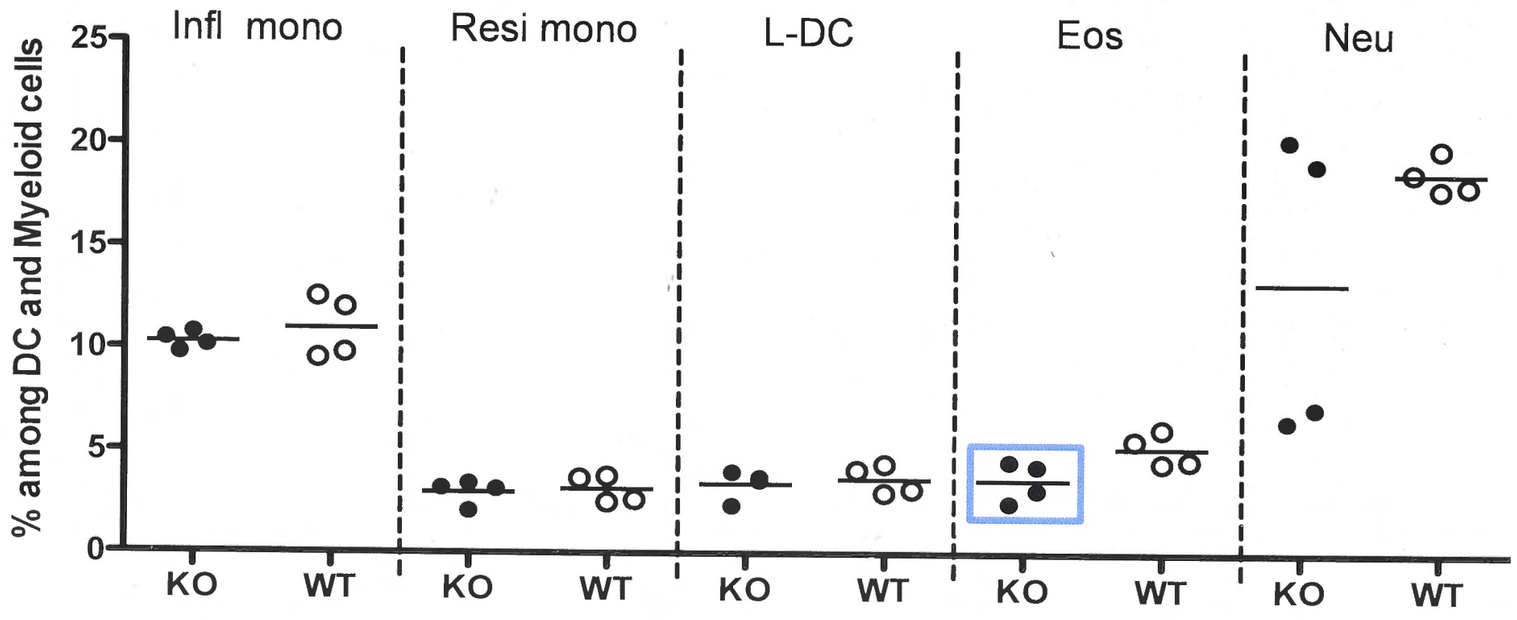
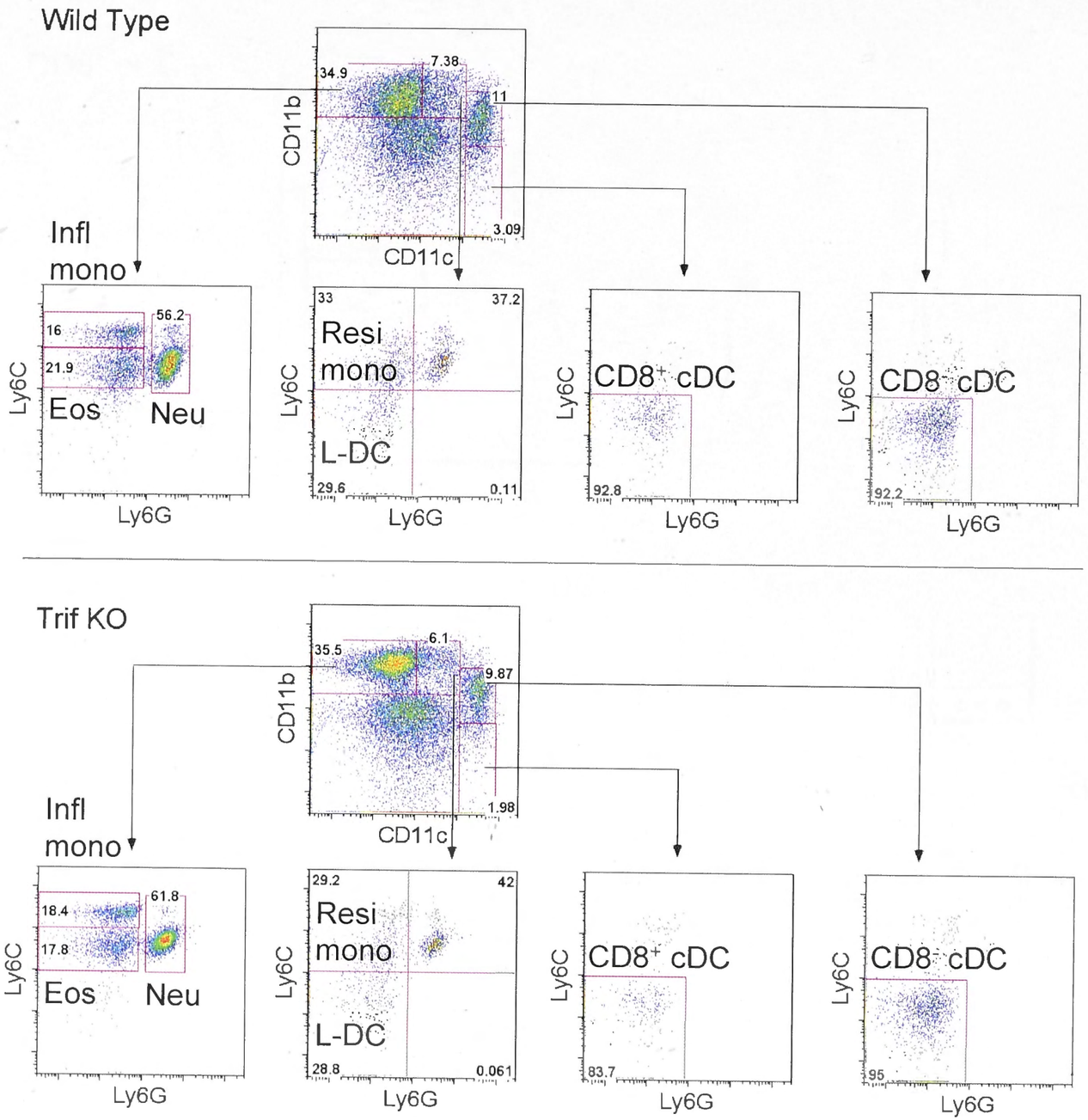

Infl mono
Resi mono
L-DC
Eos
Neu
% among DC and Myeloid cells
25
20
15
10
5
0
KO
WT
KO
WT
KO
WT
KO
WT
KO
WT

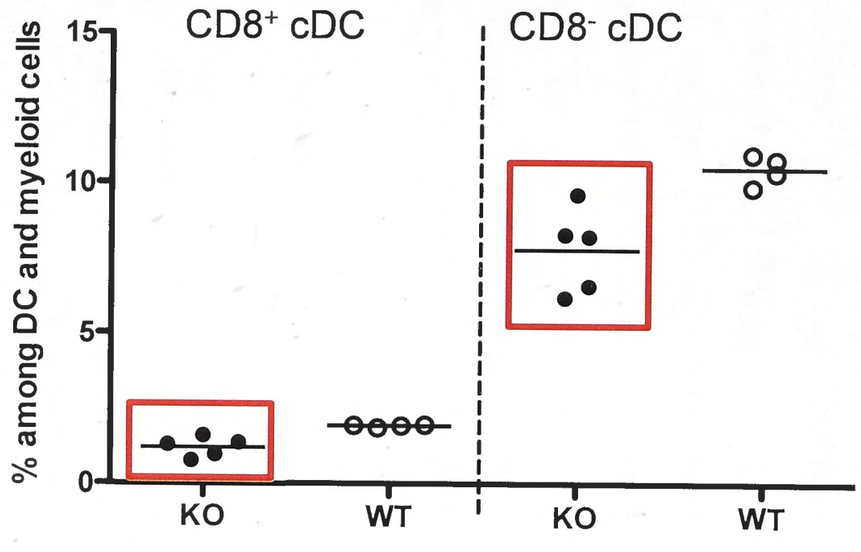
**Figure 7.4 L-DC development occurs independently of TRIF signalling.**

Splenocytes were harvested from C57BL/6J *Trif*$^{-/-}$ (TRIF KO) mice and C57BL/6J (wild type) mice, and red blood cells lysed prior to T and B cell depletion. Cells were stained with 2 distinct antibody cocktails as described in Figure 7.3. A) The gating strategy described in Figure 7.3 was also used. Data are presented as dot plots. Gates were set based on fluorescence minus one controls, and numbers in gates represent % specific binding. Individual mice were analysed. A bar is used to show mean values. B) Percent cDC is shown relative to the total dendritic and myeloid cell population. C) Percent myeloid cells and L-DC are shown relative to the total dendritic and myeloid population. Red boxes indicate a significant change relative to wild type using Student's *t*-test (p value $\leq 0.01$).

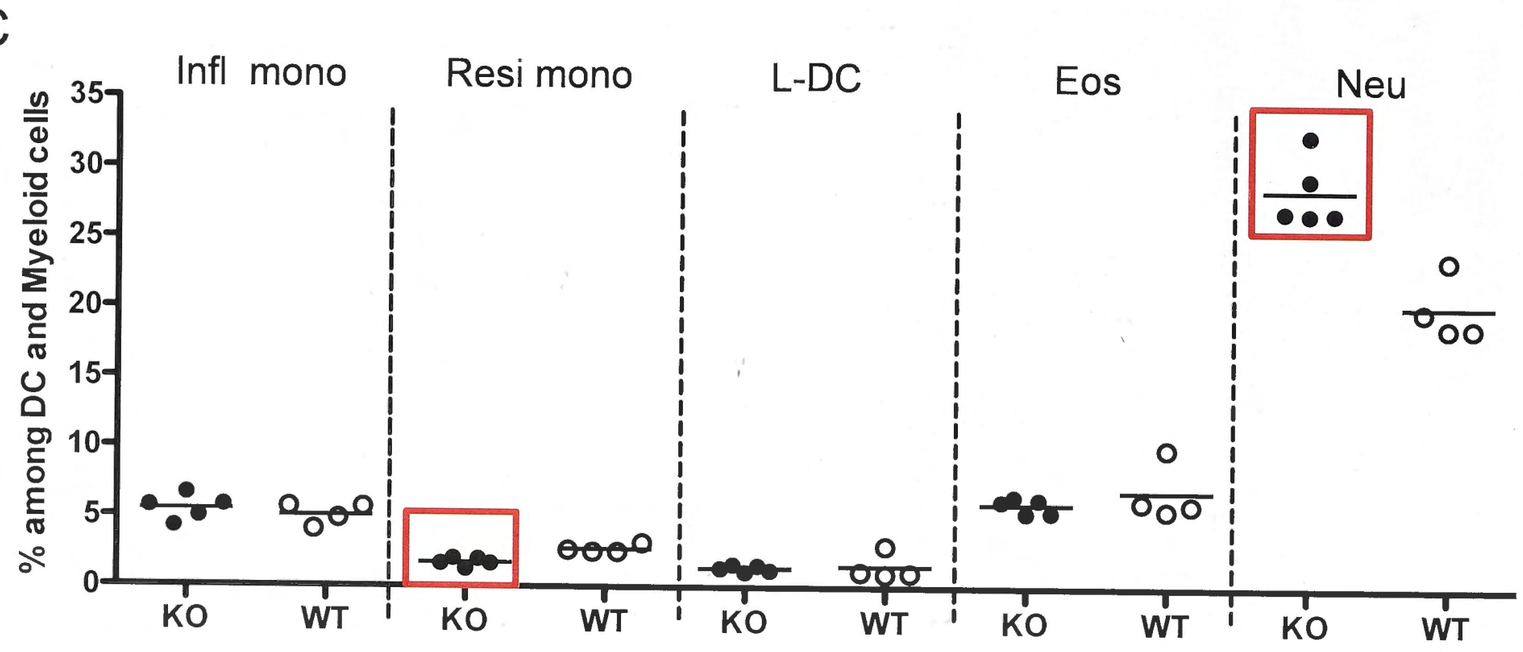
A


Wild Type
CD11b
CD11c
34.9
7.38
11
3.09
Infl mono
Ly6C
Ly6G
16
21.9
56.2
Eos
Neu
33
37.2
Resi mono
L-DC
29.6
0.11
CD8+ cDC
92.8
CD8- cDC
92.2
Trif KO
35.5
6.1
9.87
1.98
18.4
17.8
61.8
29.2
42
28.8
0.061
83.7
95

B


CD8+ cDC
CD8- cDC
% among DC and myeloid cells
15
10
5
0
KO
WT
KO
WT

C


Infl mono
Resi mono
L-DC
Eos
Neu
% among DC and Myeloid cells
35
30
25
20
15
10
5
0
KO
WT
KO
WT
KO
WT
KO
WT
KO
WT

*MyD88*$^{-/-}$/*Trif*$^{/-}$ double knockout mice were also investigated to confirm the importance of TLR signalling in splenic dendritic and myeloid cell development, *MyD88*$^{-/-}$/*Trif*$^{/-}$ mice lack all TLR. The representation of each individual subset was assessed relative to the total dendritic and myeloid cell population in wild type mice. CD8$^{-}$ cDC showed a significant drop in percentage, while there was no change in percent of CD8$^{+}$ cDC in *MyD88*$^{-/-}$/*Trif*$^{/-}$ mice (Figure 7.5 B). In addition, resident monocytes and eosinophils showed a significant drop in percentage, while inflammatory monocytes and L-DC were unaffected (Figure 7.5 C). As with *Trif*$^{/-}$ mice, neutrophils showed a significant increase in percentage in *MyD88*$^{-/-}$/*Trif*$^{/-}$ over wild type mice, a result which could reflect the infection or inflammatory status of these mice (Figure 7.5 C).

From combined studies on the three *MyD88*$^{-/-}$, *Trif*$^{/-}$ and *MyD88*$^{-/-}$/*Trif*$^{/-}$ mutants, one can conclude that there is a role for TLR signalling in the development of CD8$^{-}$ cDC, CD8$^{+}$ cDC, eosinophils and resident monocytes. However, the development of L-DC and inflammatory monocytes appears to have occurred independently of TLR signalling. The latter two subsets must develop in steady-state spleen without TLR signalling. It is important to consider that our protocol for delineation of the CD8$^{-}$ cDC subset could also capture inflammatory or monocyte-derived Tip DC whose development would be lost in *Tlr*$^{-/-}$ mice. Again, L-DC development occurs independently of inflammatory signals, and this serves to distinguish these cells as a subset distinct from resident monocytes.

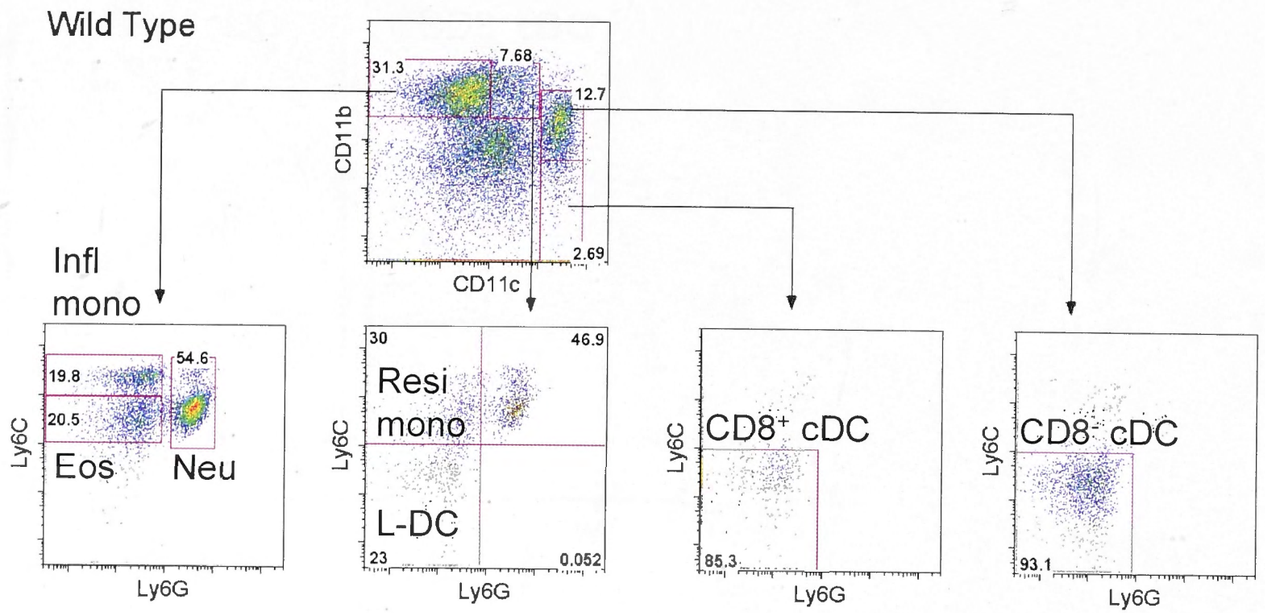
### 7.2.3 The lineage origin of L-DC is distinct from cDC

The above studies suggest that L-DC development occurs independently of growth factors and environmental factors that drive cDC development. However, it is still not known whether L-DC and cDC derive from a common lineage progenitor. To further distinguish L-DC development from cDC, their prevalence was investigated in *Batf3*$^{-/-}$ mice which have a mutation in a transcription factor expressed by cells of the cDC lineage. *Batf3* expression has been described in pre-cDC, CD8$^{+}$ cDC and CD8$^{-}$ cDC, with peak expression in differentiated cDC (Hildner et al., 2008; Murphy et al., 2013; Satpathy et al., 2012). *Batf3*$^{-/-}$ mice have been shown to contain decreased numbers of CD8$^{+}$ cDC, but not CD8$^{-}$ cDC (Hildner et

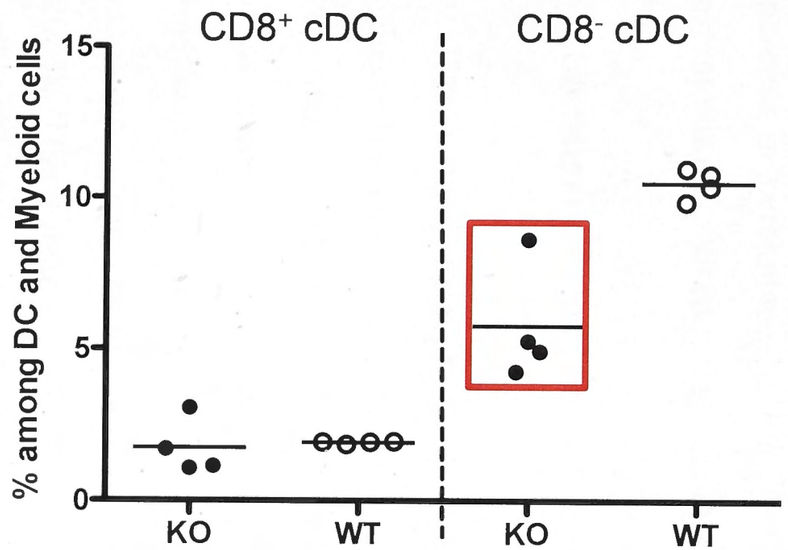
**Figure 7.5 L-DC development occurs independently of TLR signalling.**

Splenocytes were harvested from C57BL/6J *MyD88*$^{-/-}$*TRIF*$^{-/-}$ (MyD88/Trif KO) mice and C57BL/6J (wild type) mice, and red blood cells lysed prior to T and B cell depletion. Cells were stained with 2 distinct antibody cocktails as described in Figure 7.3. A) The gating strategy described in Figure 7.3 was also used. Gates were set based on fluorescence minus one controls, and numbers in gates represent % specific binding. Individual mice were analysed. A bar is used to show mean values. B) Percent cDC subsets are shown relative to the total dendritic and myeloid cell population. C) Percent myeloid cells and L-DC are shown relative to the total dendritic and myeloid population. Red boxes indicate a significant change relative to wild type using Student's *t*-test (p value $\leq 0.01$). Blue boxes indicate a significant change relative to wild type using Student's *t*-test (p value $\leq 0.05$).

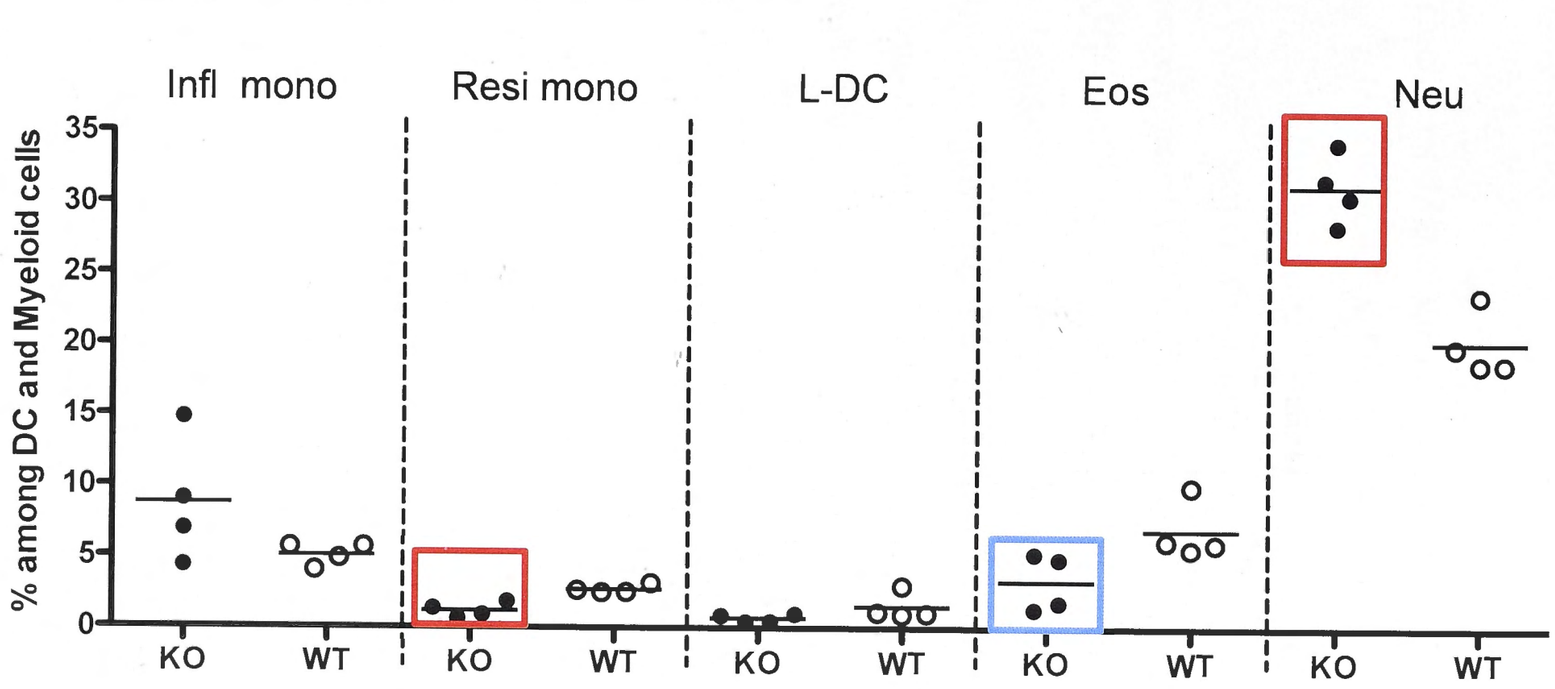
A


Wild Type
31.3
7.68
12.7
2.69
CD11b
CD11c
Infl mono
19.8
54.6
20.5
Eos
Neu
Ly6C
Ly6G
30
46.9
Resi mono
L-DC
23
0.052
CD8+ cDC
85.3
CD8- cDC
93.1
Trif/MyD88 KO
32.9
5.29
10
2
13.5
69.5
15.8
20.9
54.4
24.7
0
86.2
94.1

B


CD8+ cDC
CD8- cDC
% among DC and Myeloid cells
15
10
5
0
KO
WT
KO
WT

C


Infl mono
Resi mono
L-DC
Eos
Neu
% among DC and Myeloid cells
35
30
25
20
15
10
5
0
KO
WT
KO
WT
KO
WT
KO
WT
KO
WT

al., 2008), and *Batf3* appears to be essential for $CD8^+$ cDC development from pre-cDC (Hildner et al., 2008). *Batf3* may co-operate with another factor to induce the final differentiation of pre-cDC to $CD8^+$ cDC, while $CD8^-$ cDC differentiation may occur independently of *Batf3* (Murphy et al., 2013).

Consistent with the literature, a significant drop in the percent of $CD8^+$ cDC was observed compared with wild type mice (Figure 7.6 C). The percentage of inflammatory monocytes also dropped significantly in $Batf3^{-/-}$ mice (Figure 7.6 D). However, the percentage of $CD8^-$ cDC in $Batf3^{-/-}$ varied with four of the ten $Batf\ 3^{-/-}$ mice studied displaying an increase in percentage of $CD8^-$ cDC, while another four mice showed a lower percentage of $CD8^-$ cDC compared with controls. The variability in $CD8^-$ cDC percentage could suggest either multiple interaction effects leading to highly variable numbers, or heterogeneity amongst the $CD8^-$ cDC subset delineated here. Both L-DC and resident monocytes displayed no changes in percentage due to the *Batf3* mutation. Eosinophils were the only cells that displayed an increase in percentage in $Batf3^{-/-}$ mice. The increase in eosinophils would be consistent with increased inflammation in these mice.

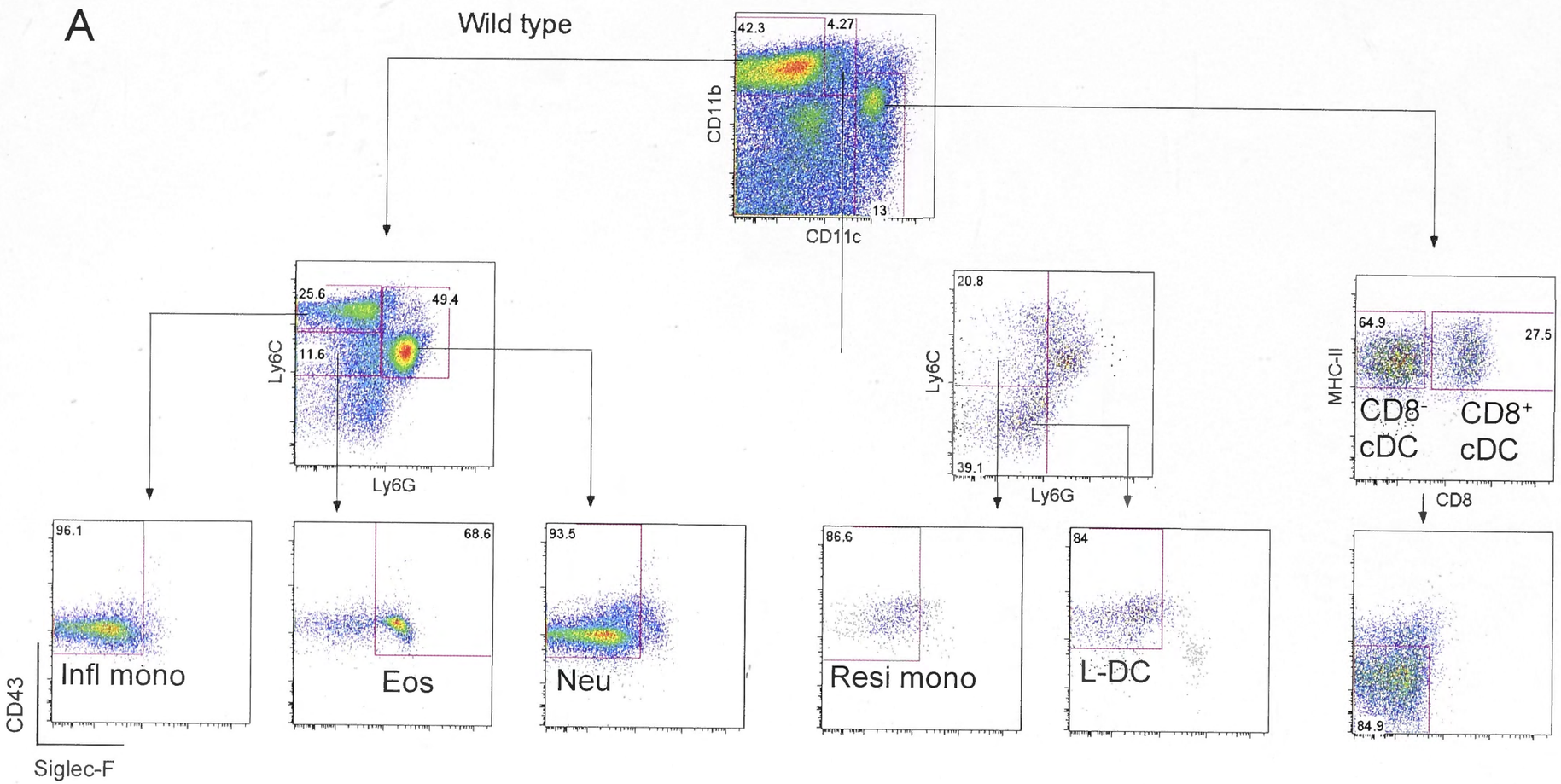
### 7.2.4 Investigation of the role of *c-Myb* in L-DC development

HSC can give rise to all blood cells, including cells of myeloid and lymphocyte lineages. Conventional DC develop from pre-cDC that derive from CDP in bone marrow, while monocytes develop from CMP in BM which traverse into blood as monocytes. Once monocytes enter tissues, they differentiate to give macrophages. Recent studies report a separate lineage of macrophages derived from yolk sac progenitors, and distinct from bone marrow-derived macrophages (Schulz et al., 2012). This lineage of macrophages is dependent on the *PU.1* transcription factor, while macrophages arising from HSC progenitors in bone marrow are dependent on the *c-Myb* transcription factor (Schulz et al., 2012). To investigate the relationship between L-DC and macrophages, a *c-Myb* mutant mouse model, Booreana ($c\text{-}Myb^{E308G}$), was analysed (Papathanasiou et al., 2010). While most mutations of *c-Myb* are embryonically lethal, this single nucleotide mutation $c\text{-}Myb^{E308G}$ allows mice to survive several weeks post birth (Papathanasiou et al., 2010). They do, however, have multiple changes amongst the hematopoetic cell

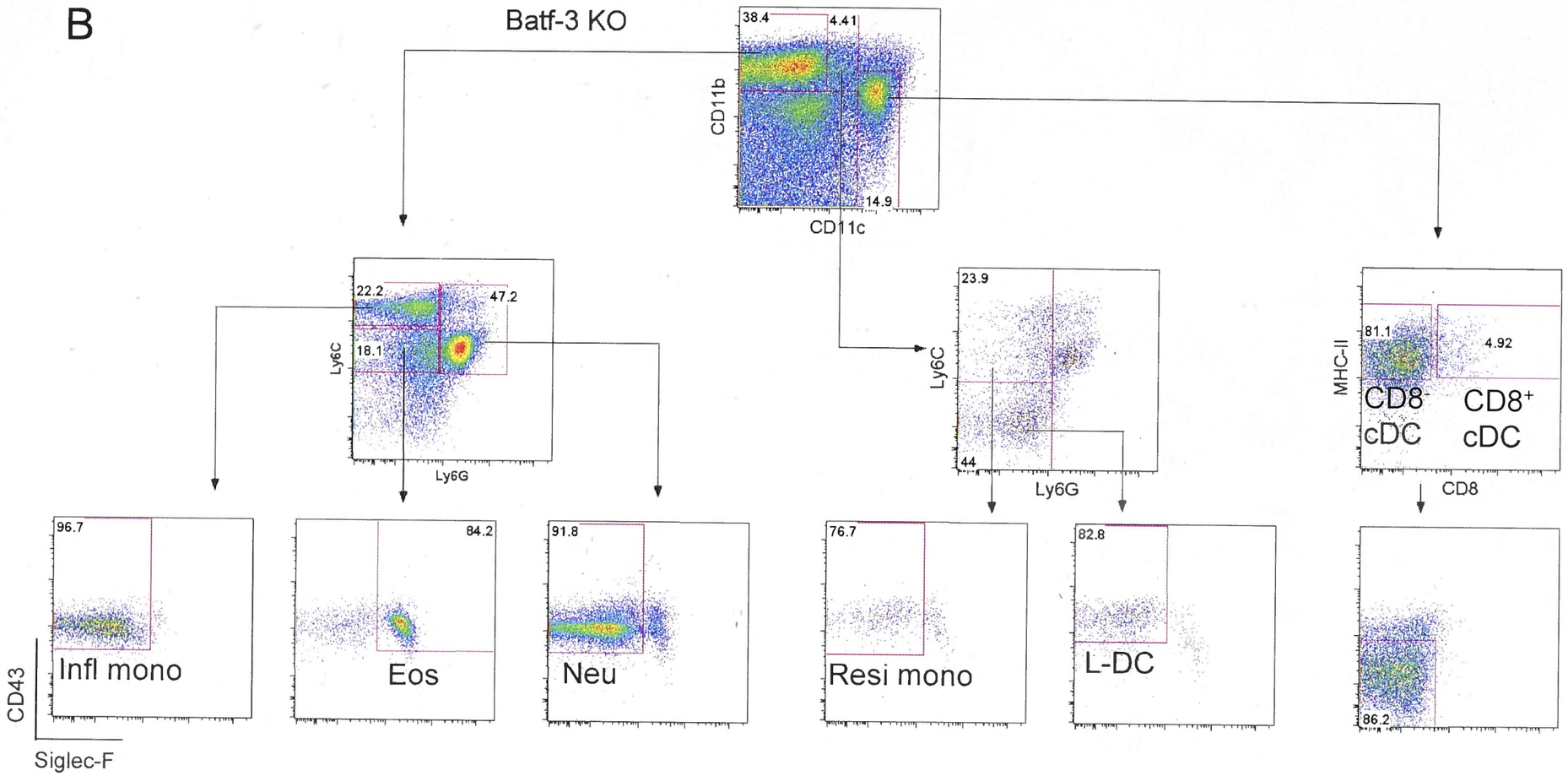
**Figure 7.6 L-DC development occurs independently of Batf-3 signalling.**

Splenocytes were harvested from B6.129S(C)-*Batf3*$^{tm1.1Kmm}$ (Batf-3 KO) and C57BL/6J (wild type) mice, and red blood cells lysed prior to T and B cell depletion. Cells were stained with 2 distinct antibody cocktails. The first included antibodies to CD11b (M1/70, Pe-Cy7), CD11c (N418, APC), Ly6C (Al-21, PB), Ly6G (1A8, PE), MHC-II (25-9-17, Biotin) and CD8 (53-6.7, FITC). The second contained antibodies to CD11b (M1/70, Pe-Cy7), CD11c (N418, APC), Ly6C (Al-21, PB), Ly6G (1A8, Bio), CD43 (IBII, FITC) and Siglec-F (E50-2440, PE). Prior to analysis, cells were stained with propidium iodide (PI, 1μg/ml) for gating live (PI$^-$) cells. A) The gating strategy to distinguish myeloid and DC subsets is shown. Myeloid cells were initially gated as CD11b$^{hi}$CD11c$^-$ cells, and further delineated into inflammatory monocytes (Infl mono: Ly6C$^{hi}$Ly6G$^-$CD43$^+$Siglec-F$^-$ cells), eosinophils (Eos: Ly6C$^+$Ly6G$^-$CD43$^+$Siglec-F$^{hi}$ cells) and neutrophils (Neu: Ly6C$^+$Ly6G$^+$CD43$^+$Siglec-F$^-$ cells). L-DC were gated as CD11b$^{hi}$CD11c$^{lo}$Ly6C$^-$Ly6G$^-$CD43$^+$Siglec-F$^-$ cells, while resident monocytes were gated as CD11b$^{hi}$CD11c$^{lo}$Ly6C$^+$Ly6G$^-$CD43$^{hi}$Siglec-F$^-$ cells. Both CD43 and Siglec-F staining were not shown. CD8$^+$cDC were gated as CD11b$^-$CD11c$^{hi}$MHCII$^+$CD8$^+$Ly6C$^-$Ly6G$^-$ cells, while CD8$^-$cDC were gated as CD11b$^+$CD11c$^{hi}$MHCII$^+$CD8$^-$Ly6C$^-$Ly6G$^-$ cells. Gates were set based on fluorescence minus one controls, and numbers in gates represent % specific binding. Individual mice were analysed. A bar is used to show mean values. B) Percent cDC subsets are shown relative to the total dendritic and myeloid cell population. C) Percent myeloid subsets and L-DC are shown relative to the total dendritic and myeloid cell population. Red boxes indicate a significant change relative to wild type using Student's *t*-test (p value $\leq 0.01$).

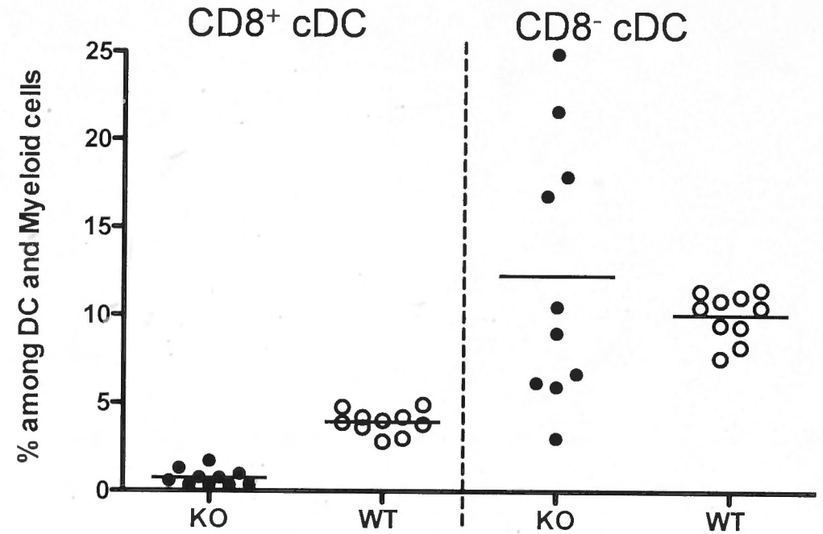
A


Wild type
42.3
4.27
13
CD11b
CD11c
25.6
49.4
11.6
Ly6C
Ly6G
20.8
39.1
Ly6C
Ly6G
64.9
27.5
MHC-II
CD8-
cDC
CD8+
cDC
CD8
96.1
Infl mono
68.6
Eos
93.5
Neu
86.6
Resi mono
84
L-DC
84.9
CD43
Siglec-F

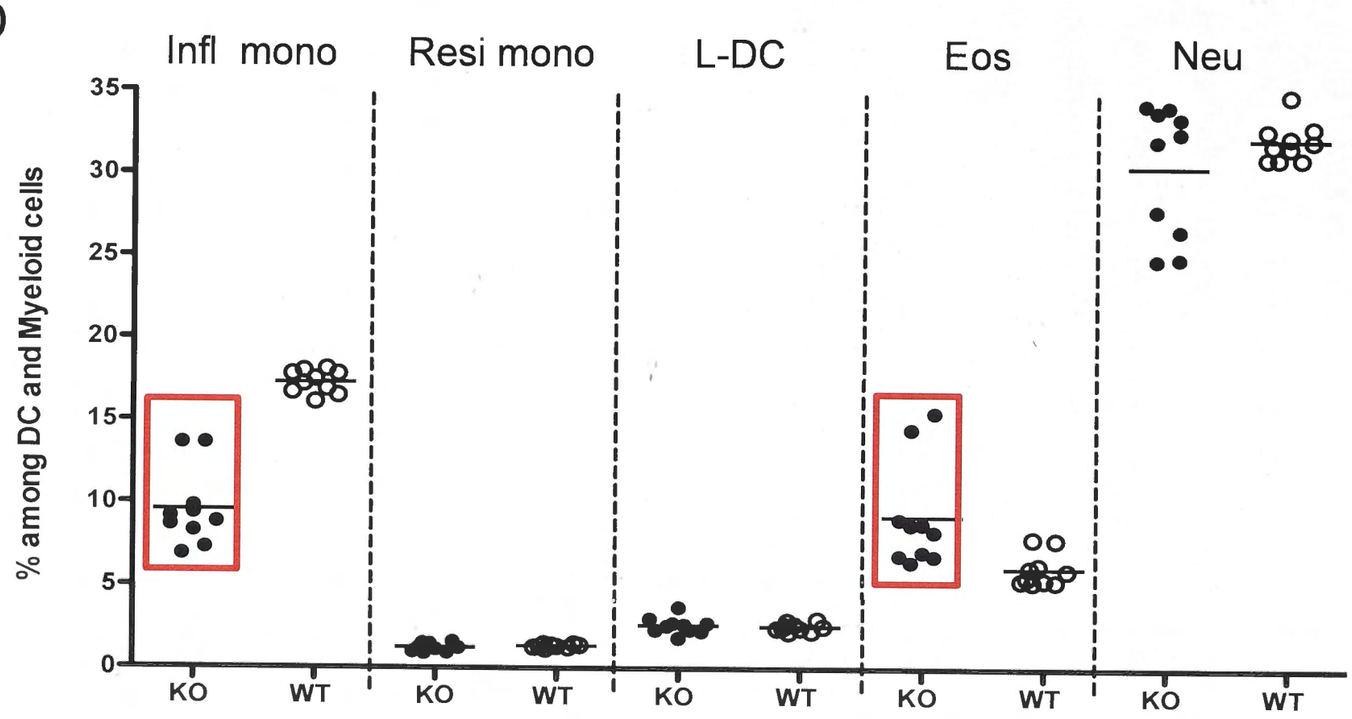
B


Batf-3 KO
38.4
4.41
14.9
CD11b
CD11c
22.2
47.2
18.1
Ly6C
Ly6G
96.7
Infl mono
84.2
Eos
91.8
Neu
CD43
Siglec-F
23.9
Ly6C
44
Ly6G
76.7
Resi mono
82.8
L-DC
81.1
4.92
MHC-II
CD8⁻ cDC
CD8⁺ cDC
CD8
86.2

C


CD8+ cDC
CD8- cDC
% among DC and Myeloid cells
25
20
15
10
5
0
KO
WT
KO
WT

D


Infl mono
Resi mono
L-DC
Eos
Neu
% among DC and Myeloid cells
35
30
25
20
15
10
5
0
KO
WT
KO
WT
KO
WT
KO
WT
KO
WT

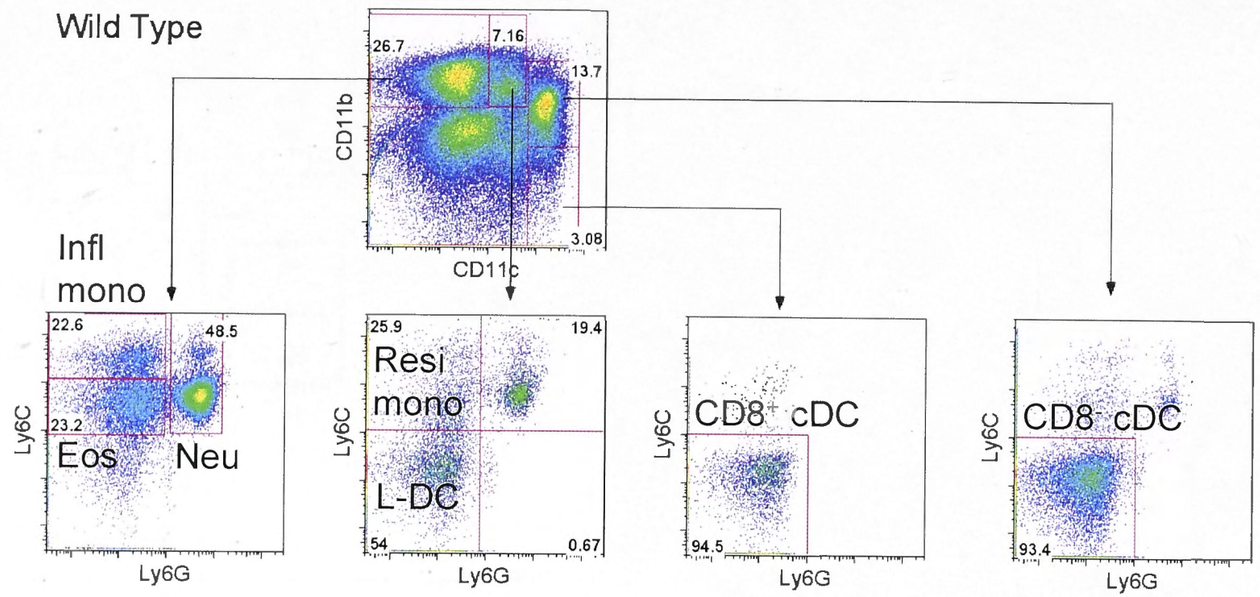
compartment, including an increase in the number of HSC (Papathanasiou et al., 2010).

Mutation in *c-Myb* resulted in a significant drop in the percentage of $CD8^-$ cDC, inflammatory monocytes and neutrophils in Booreana mice over wild type mice (Figure 7.7B). L-DC, resident monocytes and eosinophils were not affected by the *c-Myb* mutation. The explanation for this result could depend on the role of *c-Myb* in development of myeloid cells and DC from bone marrow-derived progenitors. The possibility that L-DC and resident monocytes do not derive from bone marrow progenitors but from progenitors laid down during embryogenesis, must be considered. However, an alternative explanation is needed for results observed for $CD8^+$ cDC and eosinophil development.

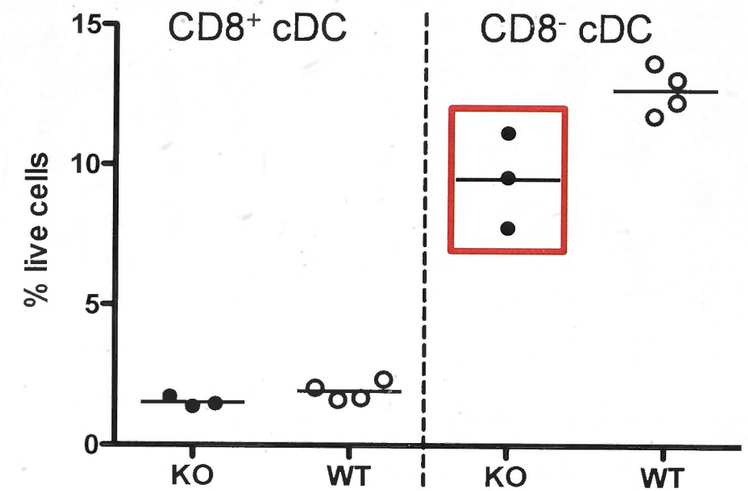
**Figure 7.7 L-DC development occurs independently of *c-Myb* signalling.**

Splenocytes were harvested from C57BL/6J*c-Myb*$^{E308G}$ (Booreana) mice and C57BL/6J (wild type) mice, respectively, and red blood cells lysed prior to T and B cell depletion. Cells were stained with 2 distinct antibody cocktails as described in Figure 7.3. A) The gating strategy in Figure 7.3 was also used. Gates were set based on fluorescence minus one controls, and numbers in gates represent % specific binding. Individual mice were analysed. A bar is used to show mean values. B) Percent cDC relative to live cells is shown. C) Percent myeloid cells and L-DC are shown relative to live cells. Red boxes indicate a significant change relative to wild type using Student's *t*-test (p value $\leq 0.01$).

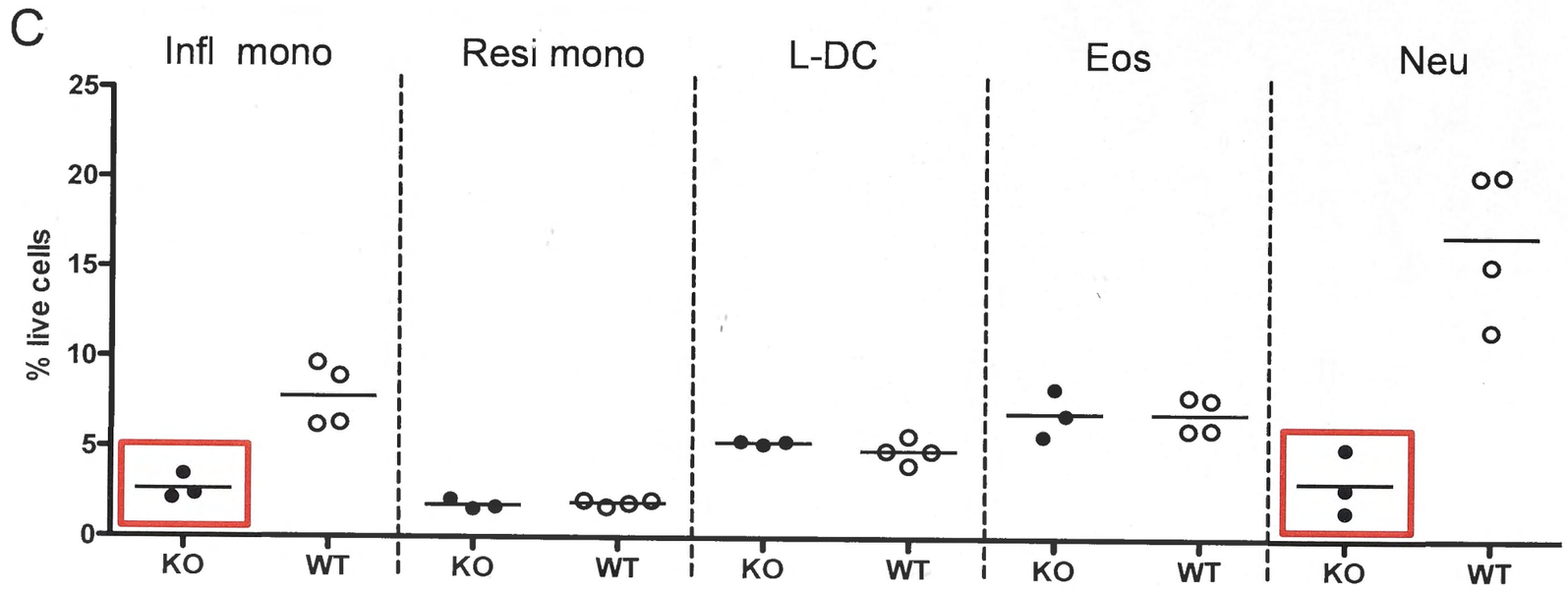
A


Wild Type
26.7
7.16
13.7
3.08
CD11b
CD11c
Infl mono
22.6
48.5
23.2
Eos
Neu
Ly6C
Ly6G
25.9
19.4
Resi mono
L-DC
54
0.67
CD8+ cDC
94.5
CD8- cDC
93.4
Booreana
10.9
7.5
17.2
3.8
14.6
14.9
54.9
28.1
13.9
56.5
1.65
98.5
92.1

B


CD8+ cDC
CD8- cDC
% live cells
15
10
5
0
KO
WT
KO
WT

C


Infl mono
Resi mono
L-DC
Eos
Neu
% live cells
25
20
15
10
5
0
KO
WT
KO
WT
KO
WT
KO
WT
KO
WT

## 7.3 Discussion

None of the mutant mice studied here are embryonically lethal, and none of the genes mutated are crucial for survival. For this reason, these strains could provide important information about the development of L-DC in relation to other splenic dendritic and myeloid subsets. L-DC development occurs independently of factors that regulate cDC and monocyte development, including FLT3L and GM-CSF. In addition, L-DC development occurs in the steady-state, independently of TLR signalling. Lastly, there is also evidence suggesting that L-DC may arise from yolk sac-derived c-*Myb*-independent progenitors in spleen, rather than from bone marrow-derived progenitors. All of these data separate L-DC from cDC as a separate lineage of cells, and identifies them as distinct from splenic monocyte subsets.

The binding of FLT3L to FLT3 triggers the development of cDC from pre-cDC (Kingston et al., 2009; Waskow et al., 2008; Xu et al., 2007). Thus, knockout of either *Flt3* and *Flt3L* adversely affects cDC development (Kingston et al., 2009; Waskow et al., 2008). Consistent with this, a significant drop in the percentage of splenic $CD8^+$ cDC and $CD8^-$ cDC was observed in $Flt3L^{-/-}$ mice (Kingston et al., 2009; Xu et al., 2007). FLT3 is also a marker of CLP and CMP (Karsunky et al., 2003), such that the delivery of additional FLT3L to mice can lead to an expansion of the monocyte pool as well as cDC (Beaudin et al., 2014; Karsunky et al., 2003). Expression of the FLT3 receptor on monocytes, and the expansion of monocytes following FLT3L stimulation, suggest that FLT3 receptor tyrosine kinase signalling plays a role in monocyte development. In agreement with this, Waskow et al. (2008) showed an increase in the numbers of MDP which give rise to CDP and monocytes following FLT3L stimulation *in vivo* and *in vitro*. A drop in the number of resident and inflammatory monocytes in $Flt3L^{-/-}$ mice shown here therefore supports a role for FLT3 signalling in monocyte development. Since L-DC development is not compromised in $Flt3L^{-/-}$ mice, L-DC is distinguishable in terms of development and lineage from both cDC subsets, as well as from resident and inflammatory monocytes in spleen.

Both FLT3L and GM-CSF have been used in different culture systems to generate DC *in vitro*. Previously, it was shown that the culture of BM precursors

with FLT3L generated cDC and pDC, while GM-CSF addition generated inflammatory type DC (Berthier et al., 2000; Kingston et al., 2009). *GM-CSF*$^{-/-}$ mice were employed here to determine any role for GM-CSF in the development of L-DC. Deficiency in GM-CSF production did not affect the development of L-DC, suggesting that L-DC are not likely to be inflammatory type DC dependent on GM-CSF signalling. By comparison, the percentage of both resident and inflammatory monocytes was reduced, consistent with their development in response to GM-CSF, and probably inflammation. However, this evidence does not necessarily mean they derive from a common progenitor. The percentage of inflammatory monocytes dropped by 4-fold in *GM-CSF*$^{-/-}$ mice, while resident monocytes showed a smaller, but significant 1.4-fold drop. Previous studies in *GM-CSF*$^{-/-}$ mice showed no significant changes in peripheral blood myeloid subsets, although alveolar macrophages were defective (Stanley et al., 1994). However, that study did not investigate splenic myeloid subsets in the same detail as used here.

GM-CSF is secreted in response to inflammation and can induce inflammatory DC or Tip DC (Berthier et al., 2000; Inaba et al., 1993; Xu et al., 2007). In contrast, cDC are generated in the steady-state, and their development occurs independently of GM-CSF (Kingston et al., 2009). Here, however, the percentage of $CD8^+$ cDC and $CD8^-$ cDC was found to be significantly increased in *GM-CSF*$^{-/-}$ mice (Kingston et al., 2009). An increase in cDC could occur as a compensatory change in spleen due to a reduction in the number of monocytes. Lastly, eosinophils displayed increased numbers in *GM-CSF*$^{-/-}$ mice suggesting that the development of eosinophils occurs independently of GM-CSF. An increase in numbers of eosinophils could also reflect an inflammatory and/or allergic state in *GM-CSF*$^{-/-}$ mice.

Inflammatory signalling induced by interferons or TLR signalling can drive HSC proliferation and differentiation (Boiko and Borghesi, 2012; Nagai et al., 2006). Murine HSC have been reported to express TLR on their cell surface and can recognise pathogen-associated molecular patterns (PAMP) (Nagai et al., 2006; Zhang et al., 2005; Zhao et al., 2013). Stimulation of HSC with LPS *in vivo* leads to an increase in HSC cycling, evident by increased colony forming units in spleen, blood and BM (Zhang et al., 2005). The expression of TLR on HSC could reflect a

mechanism whereby HSC can detect infection directly, and respond by increased hematopoiesis. In order to investigate whether TLR signalling plays a role in L-DC development, the three *MyD88*$^{-/-}$, *Trif*$^{/-}$and *MyD88*$^{-/-}$/*Trif*$^{/-}$ mutant mice were studied. The combined results showed no significant change in the percentage of L-DC across all three mutants, confirming that L-DC development occurs independently of TLR signalling. In contrast, cDC subsets were reduced in TLR mutants. $CD8^+$ cDC and $CD8^-$ cDC development and homeostasis are clearly dependent on *Trif* signalling evident by a drop in the numbers of cells in *Trif*$^{/-}$ and *MyD88*$^{-/-}$/*Trif*$^{/-}$ mice. TRIF acts as an adaptor protein for TLR3 signal transduction, an essential pathway for DC recognition of virus (West et al., 2006). Hence, lack of *Trif* expression would affect cDC ability to activate T cells.

Resident monocytes also showed reduced numbers in *Trif*$^{/-}$ and *MyD88*$^{-/-}$ */Trif*$^{/-}$ mice, suggesting that their development is also dependent on TLR3 signalling. Despite distinct lineage origins, both cDC and resident monocytes share the same requirement for TLR3 signalling. However, numbers of inflammatory monocytes, which are also precursors of Tip DC (Geissmann et al., 2008), were not affected in any of the TLR mutants. The conversion of inflammatory monocytes to immature Tip DC during parasitic infection has been shown to occur independently of IFN-γ and MYD88, while the activation of immature Tip DC is dependent on both signalling pathways (Bosschaerts et al., 2010). Since inflammatory monocyte numbers are unchanged in TLR mutant mice, it would appear that all mice under study here reflect steady-state conditions, and without conversion of inflammatory monocytes to Tip DC. These studies therefore highlight how resident monocytes and inflammatory monocytes have distinct requirements for TLR signalling.

Out of all the subsets studied in TLR mutant mice, only eosinophils showed a drop in numbers in *MyD88*$^{-/-}$ mice. Eosinophils are essential for activation of innate immunity against parasite infections and allergic responses through engagement of TLR7 (Nagase et al., 2003). MYD88 is required for all TLR signalling except TLR3, thus lack of MYD88 signalling could adversely affect eosinophils. Lastly, one interpretation for the increased proportion of neutrophils observed in *Trif*$^{/-}$ and *MyD88*$^{-/-}$/*Trif*$^{/-}$ mice could be that there is a compensatory increase due to reduced numbers of resident monocytes and cDC observed in these mice.

L-DC development occurs independently of FLT3L, GM-CSF and TLR signalling. Previously, this lab published evidence that L-DC could arise from progenitors in BM, in particular LT-HSC and MPP when co-cultured over splenic stromal lines (Hinton and O'Neill, 2012; Petvises and O'Neill, 2014). These findings support mounting evidence that L-DC arise from a progenitor distinct from CDP. Data shown here are consistent, and serve to further delineate the L-DC lineage from the cDC lineage. BATF-3 is a transcription factor essential for the differentiation of CDP or pre-cDC to give $CD8^+$ cDC (Hildner et al., 2008). In *Batf-3*$^{-/-}$ mice, a significant drop in $CD8^+$ cDC development was observed consistent with the literature (Hildner et al., 2008). However, the percentage of L-DC was unaffected in *Batf-3*$^{-/-}$ mice, suggesting that L-DC have a distinct lineage origin from cDC, although a significant drop in the percentage of inflammatory monocytes was observed. Recent studies in *Batf-3*$^{-/-}$ mice showing a defect in the differentiation of CD4 T helper cells into T helper 17 (Th17) cells (Kurachi et al., 2014; Schraml et al., 2009) which are essential in conducting inflammatory responses against pathogens and in autoimmunity (Bettelli et al., 2006; Brüstle et al., 2007). It is interesting to speculate that inflammatory monocytes may be important mediators of Th17 cell development.

It is well known that a distinct lineage of macrophages arises from yolk sac-derived hematopoietic progenitors or HSC (Schulz et al., 2012). The study used $CX_3CR1^{gfp/+}$ mice, where one copy of the gene encoding $CX_3CR1$ was replaced with green fluorescent protein (GFP) reporter cDNA, to track of the development of macrophages during embryogenesis. Two populations of macrophages were observed in $CX_3CR1^{gfp/+}$ mice. $CD11b^{lo}F4/80^{hi}CX_3CR1^{hi}$ cells were first detected in the embryo between E9.5 and E10.5, while $CD11b^{hi}F4/80^{lo}CX_3CR1^{lo}$ cells were observed after E12.5. The lineage origin of these macrophages was confirmed using *PU.1*$^{-/-}$ and *c-Myb*$^{-/-}$ mutant mice (Schulz et al., 2012). PU.1 is an essential transcription factor for macrophage development, but dispensable for HSC development (Dakic et al., 2005), while C-MYB is an essential transcription factor for hematopoietic development, but is dispensable for macrophage development (Mucenski et al., 1991; Mukouyama et al., 1999; Sumner et al., 2000). $CD11b^{lo}F4/80^{hi}CX_3CR1^{hi}$ cells were present in c-*Myb*$^{-/-}$ mice, but absent in *PU.1*$^{-/-}$

mice, indicative of a yolk sac origin. In contrast, $CD11b^{hi}F4/80^{lo}CX_3CR1^{lo}$ cells were present *PU.1*$^{-/-}$ mice, but absent in c-*Myb*$^{-/-}$ mice, indicative of a bone marrow origin (Schulz et al., 2012). This study raises the question of whether other cell types also derive from yolk sac-derived progenitors. Previous evidence indicates that L-DC derive from HPC which are endogenous to spleen, which seed spleen during embryogenesis (O'Neill et al., 2014).

In order to investigate the lineage origin of L-DC as either bone marrow- or yolk sac- derived, *Booreana* mice (*c-Myb*$^{E308G/E308G}$) were employed (Papathanasiou et al., 2010). Increased numbers of HSC, MPP, CLP and CMP and a drop in GMP and MEP numbers have been recorded in *Booreana* bone marrow (Papathanasiou et al., 2010). These changes are also reflected in progeny cells in blood, and significant reduction in the numbers of neutrophils and red blood cells were also observed. Here it was shown that spleens of *Booreana* mice carry reduced numbers of $CD8^-$ cDC, inflammatory monocytes and neutrophils, consistent with their origin from bone marrow progenitors, and consistent with decreased numbers of CMP and GMP in *Booreana* bone marrow (Petvises and O'Neill, unpublished data). However, resident monocytes and L-DC were present in similar numbers in *Booreana* and wild type mice, suggesting that both resident monocyte and L-DC development occur independently of *c-Myb*. These findings support the possibility that L-DC and resident monocytes in spleen arise from endogenous yolk sac-derived hematopoietic progenitors. Whether they are related in terms of lineage is not yet known.

None of the mutants studied here showed any effect on L-DC development. Clearly the development of L-DC is distinct from cDC, monocytes, eosinophils and neutrophils. In contrast, cDC development and homeostasis was affected by signalling dependent on *Flt3*, *Batf-3* and *TLR3* signalling. The development and homeostasis of both resident monocytes and inflammatory monocytes were adversely affected by mutations affecting *Flt3* and *GM-CSF* signalling. In addition, the production of inflammatory monocytes was shown to be dependent on *Batf-3* and *c-Myb* signalling, while resident monocytes were dependent on *Trif* signalling.

# Chapter 8

# General discussion

## 8.1 Identification of L-DC in relation to other splenic dendritic and myeloid cell subsets

In general terms, this thesis has made a number of contributions towards better understanding of the dendritic and myeloid subsets present in murine spleen. In particular, the novel dendritic-like subset 'L-DC' has been further characterised in relation to other known dendritic cell (DC) subsets as well as monocytes. New procedures used for characterisation of subsets have been verified, in that the $CD8^+$ cDC and $CD8^-$ cDC subsets can be clearly identified, and are shown here to resemble the cDC subsets described by others. L-DC are shown as clearly distinct from cDC subsets. Full and complete analysis of splenic subsets has required that the monocyte subsets be redefined, with the outcome that inflammatory monocytes are now clearly distinguishable as a separate lineage from resident monocytes, and are also distinct from L-DC. The resident monocyte subset defined here as a subset of spleen has been shown to be clearly distinct from described resident monocytes present in murine blood. Resident monocytes are also shown to be distinct as a subset from L-DC, although there is clear evidence that these subsets are similar, and probably derived from a common progenitor or lineage. L-DC are therefore defined as a separate novel dendritic-like subset in spleen as previously hypothesised (O'Neill et al., 2014).

This study has used flow cytometry extensively to better identify the cell surface marker phenotype of subsets (Chapters 3 and 4). L-DC is now best distinguished as a $CD11b^{hi}CD11c^{lo}MHCII^-Ly6C^-Ly6G^-$ subset of cells, which are also $CX3CR1^{lo}CD43^{lo}Siglec\text{-}F^-$. They are distinct from eosinophils on the basis of $CD11c^{lo}$ expression and lack of Ly6C and Siglec-F expression. In terms of their relationship to monocyte subsets, L-DC are distinct from resident monocytes on the basis of their $CX_3CR1^{lo}Ly6C^-CD115^-$ phenotype, while resident monocytes are $CX_3CR1^{hi}Ly6C^{lo}CD115^+$ cells. L-DC are also distinct from inflammatory monocytes on the basis of $CD11c^{lo}Ly6C^-$ expression, while inflammatory monocytes are $CD11c^-Ly6C^{hi}$ cells. They are clearly distinct from cDC in that they lack MHCII expression, and also on the basis of CD43 and $CX_3CR1$ expression. Further tests of L-DC function, gene expression and lineage have therefore been based on a new set of distinguishing markers (Chapter 5). Functional assays used to distinguish the splenic

dendritic and myeloid subsets have been limited by low cell yields achieved after cell sorting, and by the low representation of DC subsets in spleen. In terms of characterising L-DC as an *in vivo* equivalent of LTC-DC, ability to cross-present antigen was investigated primarily. Initially, the endocytic ability of L-DC for soluble antigen and receptor-mediated antigen uptake was confirmed. L-DC demonstrated similar ability as $CD8^+$ cDC to take up antigen via mannose receptors, and weaker ability to endocytose soluble OVA compared with the cDC subsets. Initially, several protocols for assessment of cross-presentation were tested, including *in vitro* pulsing of DC with antigen and *in vivo* priming. However, cell numbers recovered were too low and this compromised experimentation (data not shown). For this reason, mACT-OVA mice were employed which express cell surface antigen (OVA) constitutively. These experiments confirmed the cross-presentation ability of $CD8^+$ cDC and $CD8^-$ cDC, but not of neutrophils, resident and inflammatory monocytes, as shown previously by others. An established experimental procedure was therefore proven reliable in identifying cross-presentation as a property of the more clearly characterised L-DC subset. L-DC showed weaker ability in cross-presentation than cDC, but regularly showed cross-presentation ability across multiple experiments.

Mutant mice were utilised to confirm the development and lineage origin of L-DC as distinct from cDC and monocyte subsets (Chapter 7). The development of L-DC occurred independently of growth factors like FLT3L and GM-CSF, known to be essential for cDC and monocyte/macrophage development, respectively. L-DC also develop independently of BATF3, a transcription factor required for $CD8^+$ cDC development. The development of L-DC also occurs independently of inflammatory signals, consistent with a subset resident in spleen under steady-state conditions. L-DC develop independently of TLR signalling, and this was also shown to be true for inflammatory monocytes. In contrast, the splenic cDC and resident monocytes were found to be dependent on TLR3 and TLR4 signalling, consistent with inflammation as a factor in the development of at least some cells in these subsets.

In terms of their restricted development in only spleen, the question of L-DC origin from a spleen versus a bone marrow progenitor was addressed. The development of dendritic and myeloid subsets from bone marrow progenitors has

been shown to be critically dependent on *c-Myb*. The development of L-DC was therefore analysed in *c-Myb*$^{E308G}$ mutant mice which show reduced hematopoiesis in bone marrow with subsequent changes in blood leukocyte numbers. Mice containing the non-lethal *c-Myb*$^{E308G}$ mutation showed reduced production of neutrophils, inflammatory monocytes and CD8$^-$ cDC in spleen. The production of resident monocytes and L-DC was however unaffected by this mutation, consistent with L-DC and resident monocytes having a developmental origin distinct from bone marrow progenitors. The possibility that they derive from a spleen endogenous progenitor laid down during embryogenesis, and perhaps of yolk sac origin, has been considered. These data also support a common lineage relationship and progenitor origin for L-DC and resident monocytes in spleen, despite their clear phenotypic and functional differences.

Transcriptome analysis was used initially to investigate the incompletely characterised L-DC subset in relation to other well defined subsets of cDC and granulocytes (Chapter 3). This initial study identified L-DC as functionally similar to DC, but also bearing myeloid characteristics. L-DC were clearly distinguishable from cDC. This initial study also identified contaminant cell populations and contributed to the development of optimised procedures for delineation of subsets on the basis of marker analysis. A subsequent transcriptome analysis in Chapter 6 involved the more fully defined subsets identified in Chapter 4. This allowed cDC subsets, inflammatory monocytes and eosinophils to be clearly distinguished as pure subsets and distinct from L-DC. This study showed clearly that a newly defined resident monocyte subset was very similar to L-DC. However, both subsets showed clear differences in their function as cross-presenting cells, with only L-DC and not resident monocytes effective in cross-presentation and in activation of CD8$^+$ T cells in response to antigen. L-DC and resident monocytes were also clearly distinct in terms of their development. Numbers of resident monocytes but not L-DC were compromised in *Trif*$^{-/-}$, *Flt3L*$^{-/-}$ and *Gm-csf*$^{-/-}$ mice, consistent with a role for inflammation in the formation of resident monocytes but not L-DC. In terms of their transcriptome, L-DC reflect a distinct novel dendritic-like cell type, showing expression of genes which drive antigen presentation as well as activation and regulation of T cells, consistent with a dendritic antigen presenting cell phenotype.

However, L-DC also express genes reflective of the myeloid lineage which are commonly expressed by both the resident and inflammatory monocyte subsets.

## 8.2 Redefining the splenic myeloid subsets

While it has been known for a long time that the spleen contains multiple dendritic and myeloid subsets, with different subsets distinguishable on the basis of phenotype, function or anatomical location, the relationship between subsets has not been clearly defined. As a result of recent intensive flow cytometric analysis, splenic DC subsets are now better characterised than the myeloid subsets. These remain in need of better delineation. For the myeloid subsets, the literature contains multiple descriptions of different phenotype and function. The history of this work led to confusion in the identification of splenic dendritic subsets for some time, and now remains a problem for the definition of myeloid subsets. Indeed, the same cell type could be named differently on the basis on function versus phenotype. For example, in the gut, multiple DC subsets can be identified based on CD11b, CD8, CD103 and CX3CR1 expression (Iwasaki and Kelsall, 2001; Johansson-Lindbom et al., 2005; Scott et al., 2011; Varol et al., 2009). The $CD103^{+}CX_3CR1^{-}$ and $CD103^{-}CX_3CR1^{+}$ DC subsets in lamina propria induce tolerogenic and pro-inflammatory responses, respectively (Atarashi et al., 2008; Denning et al., 2007). The $CD103^{-}CX3CR1^{+}$ DC subset has however been described as a macrophage on the basis of developmental origin (Bain and Mowat, 2014), but as a DC on the basis of CD11c and MHCII expression (Scott et al., 2011; Varol et al., 2009). In order to address these issues, a recent paper suggested a unified nomenclature for DC, monocytes and macrophages based primarily on ontogeny, and secondly in terms of location, function and phenotype (Guilliams et al., 2014). This study defined subsets in terms of their anatomical location as well as their phenotype. A case in point in this thesis has been the delineation of resident monocytes in spleen on the basis of the published phenotype and function of resident monocytes in blood. This study revealed that the spleen equivalent subset was not a resident monocyte but instead a subset of eosinophils. Further analysis was needed to identify a candidate resident monocyte cell type in spleen. Indeed, the phenotype of subsets can vary from tissue to tissue, emphasising how important it is to identify subsets on the basis of multiple criteria and to have a unified nomenclature for dendritic and myeloid subsets.

In order to better define L-DC in relation to splenic myeloid subsets, it was necessary to identify myeloid and DC subsets concurrently. Historically, splenic monocytes have been classified as resident and inflammatory monocytes with the splenic subsets identified on the basis of the original blood subset phenotypes. Since different markers were used previously to identify blood monocytes, a combination of all known markers was incorporated into the staining protocol. Monocytes have been described previously as $CD11b^{hi}CD11c^{-}MHCII^{-}$ cells, and then further delineated into inflammatory or resident monocyte subsets (Geissmann et al., 2008; Geissmann et al., 2003). Inflammatory monocytes are commonly gated as $CX_3CR1^{lo}Ly6C^{hi}Ly6G^{-}$ cells, while resident monocytes are gated as $CX_3CR1^{hi}Ly6C^{+}Ly6G^{-}$ cells (Geissmann et al., 2008; Geissmann et al., 2003). However, in Chapter 4, it was found that gating according to this practice revealed resident monocytes as a $CD11c^{-}$ subset which did not express $CX_3CR1$. Instead, high levels of $CX_3CR1$ common to resident monocytes were expressed by a subset of $CD11c^{lo}$ cells, and restricted also to a $Ly6C^{+}Ly6G^{-}$ subset.

The expression of CD11c on monocytes has been a debatable issue, with some investigators describing cells as $CD11c^{-}$ while others as $CD11c^{lo}$ cells. More comprehensive staining for myeloid markers revealed that an apparent subset of $CD11c^{-}$ resident monocytes stained positive for Siglec-F, which is a delineating marker for eosinophils, and not resident monocytes. In addition, morphological studies in Chapter 4 confirmed the identity of $CD11b^{hi}CD11c^{-}MHCII^{-}Ly6C^{lo}Ly6G^{-}$ cells as eosinophils. In the same study, phenotypic and morphological analysis identified $CD11b^{hi}CD11c^{lo}MHCII^{-}Ly6C^{+}Ly6G^{-}$ cells as resident monocytes, distinguishing them from previously defined subsets. This newly defined resident monocyte subset, as well as a subset of inflammatory monocytes, showed similar functional characteristics as monocytes in that they expressed common markers like CCR2, CSF1R, CD44, CD209 and F13A1 (Chapters 3 and 6) (Ingersoll et al., 2010; Swirski et al., 2009). In addition, the full development of both monocyte subsets in spleen was found to be inhibited in *Flt3*$^{-/-}$ and *Gm-cfsr*$^{-/-}$ mice. These genes encode Flt3 and GM-CSFR which are important in the development of myeloid subsets (Beaudin et al., 2014; Karsunky et al., 2003; Xu et al., 2007). This study has highlighted the complexity and confusion in the nomenclature used to define DC and

myeloid subsets. Phenotype alone is insufficient to correctly identify a subset, and a combination of morphology, function and gene expression are therefore required to confirm subset identify.

## 8.3 L-DC as a novel dendritic-like subset

Antibody staining and flow cytometric analysis were used primarily to distinguish L-DC from other splenic dendritic and myeloid subsets. Initial studies identified L-DC as $CD11b^{hi}CD11c^{lo}MHCII^{-}CD8^{-}B220^{-}$ cells, distinct from cDC on the basis of phenotype (Chapter 3). Further phenotypic and morphologic studies revealed that a candidate L-DC subset is distinct from macrophages, inflammatory monocytes, resident monocytes, neutrophils and eosinophils. L-DC are distinct from $CD8^{+}$ cDC primarily by lack of MHCII and CD40 expression but also since they express CD11b, CD43 and $CX_3CR1$ (Chapters 3 and 4). MHCII expression is associated with APC and presentation of antigen to $CD4^{+}$ T cells, and CD40 is an essential co-stimulatory molecule expressed on APC for activation of T helper cells (Pooley et al., 2001; Van Essen et al., 1995). Lack of MHCII and CD40 expression is consistent with L-DC inability to present antigen to $CD4^{+}$ T cells and to induce a T helper response (Chapter 5). In contrast, L-DC showed resemblance with $CD8^{-}$ cDC in terms of expression of CD11b, $CX_3CR1$ and F4/80 (Chapters 3 and 4). However, transcriptome analysis also showed L-DC to be distinct from cDC on the basis of overall gene expression and clustering analysis. Despite these differences, L-DC resemble cDC morphologically, and can function in cross-presentation with ability to cross-prime $CD8^{+}$ T cells (Chapters 4 and 5). Indeed, L-DC reflect dendritic-like cells, showing similar ability to take up and retain mannan as $CD8^{+}$ cDC. Cross-presentation is a hallmark of DC, and distinguishes DC from other myeloid subsets. A previous study showed that $CD8^{+}$ cDC mainly utilised the TAP pathway for processing antigen for cross-presentation to $CD8^{+}$ T cells (Ming et al., 2008). However, L-DC appear to use a TAP-independent pathway, since cytochrome c treatment of cells does not inhibit their cross-presentation capacity. It is possible that L-DC could utilise both TAP-dependent and TAP-independent pathways for processing of antigen for cross-presentation under different conditions. L-DC also showed similar ability as $CD8^{+}$ cDC to induce cytotoxic T cells through specific lysis of target cells.

The possibility that L-DC reflect a macrophage subset on the basis of lack of MHCII expression and low level CD11c expression was considered, but refuted. It was first important to better define macrophages in spleen on the basis of phenotype since most studies have used immunocytochemistry to identify macrophages. Here, splenic macrophages were identified as $CD11b^{lo}CD11c^{-}Ly6C^{-/+}Ly6G^{-}$ cells through a series of staining and backgating strategies (Chapter 4). Further staining for markers which define particular splenic macrophage subsets, including SIGNR1, MOMA-1 and CD68, confirmed that L-DC is a distinct subset. L-DC has also shown to be distinct from neutrophils, eosinophils and inflammatory monocytes in terms of phenotype, morphology and gene expression. L-DC can be delineated from neutrophils by lack of Ly6G and 7/4 expression (Galli et al., 2011; Rosas et al., 2010). L-DC could be also distinguished from eosinophils by lack of Siglec-F expression (Chapter 4) (Bochner, 2009; Guo et al., 2007), and from inflammatory monocytes by absence of Ly6C expression.

Our working hypothesis is that L-DC represent an APC subset appropriately localised in spleen to present blood-borne antigen for activation of $CD8^{+}$ T cells. This hypothesis is supported by evidence for the high endocytic and cross-presentation ability of L-DC. Identification of gene expression unique to L-DC has revealed 3 specific markers related to antigen presentation including CD300E, CD300LD and CD9 (Chapter 6). Both CD300E and CD300LD are members of the CD300 family, which regulates the immune response via paired activating and inhibitory receptors (Borrego, 2013; Izawa et al., 2007). In addition, CD300 molecules have been described to bind to lipids, like phosphatidylserine and phosphatidylethanolamine, which are exposed on the plasma membrane of apoptotic and activated cells (Borrego, 2013). CD300E also participates in T cell activation via regulation of transcription factor expression (Brckalo et al., 2012), and in monocyte and mo-DC activation (Brckalo et al., 2010). It induces the release of reactive oxygen in monocytes, as well as cytokine release and expression of activation markers upon binding (Brckalo et al., 2010). CD300E-primed DC show stronger ability to activate T cells, while CD300LD induces cytokine production in neutrophils and macrophages (Izawa et al., 2007). CD9 can act as a costimulatory molecule on DC to activate T cells, and facilitates the uptake of exosomes by APC

(Kobayashi et al., 2004; Lagaudrière-Gesbert et al., 1997). These unique markers support the hypothesis that L-DC function as an APC with distinct ability to present antigen and activate T cells. Here, L-DC are identified as a novel subset of dendritic-like cells with many characteristics of myeloid lineage cells, but with antigen presenting function reflecting a subset of DC unique to spleen.

## 8.4 Relationship between L-DC and resident monocytes

Data presented here identify L-DC as closely related to splenic resident monocytes in terms of phenotype and gene expression profile, but distinct in terms of function, morphology and development. Both L-DC and resident monocytes were gated as $CD11b^{hi}CD11c^{lo}MHCII^{-}CD8^{-}Ly6G^{-}$ cells, then further distinguished on the basis of Ly6C, $CX_3CR1$ and CD115 expression (Chapters 3 and 4). L-DC were further identified as $Ly6C^{-}CX_3CR1^{lo}CD115^{-}$ cells, while resident monocytes were $Ly6C^{+}CX_3CR1^{hi}CD115^{+}$ cells (Chapters 3 and 4). The gene expression profiles of L-DC and resident monocytes were found to be very similar, suggesting that the two subsets could be derived from a common progenitor. Despite their similarity, L-DC can be distinguished from resident monocytes by cross-presentation ability and distinct morphology. Developmental studies in Chapter 7 also highlighted how resident monocytes in spleen are dependent on GM-CSF, FLT3, TRIF and TRIF/MYD88 for development, while L-DC are not. However, linkage between L-DC and resident monocytes was indicated by evidence of a possible common lineage origin since the development of both subsets remained unaffected in Booreana mice which contain a non lethal mutation in *c-Myb*. Hematopoiesis from bone marrow progenitors depends on *c-Myb* expression, so the hypothesis that L-DC and resident monocytes develop in spleen from an endogenous progenitor laid down during embryogenesis, and perhaps a yolk sac-derived progenitor, should be considered (O'Neill et al., 2014). During primitive hematopoiesis, yolk sac-derived progenitors are laid down in tissues and then generate tissue-specific macrophages after birth (Ginhoux and Jung, 2014). Recent studies have shown that microglia and Langerhans cells are derived from yolk sac-derived progenitors seeded into tissues during embryogenesis (Ginhoux et al., 2010; Merad et al., 2004; Merad et al., 2002). Yolk sac-derived macrophages can be distinguished from bone marrow-derived macrophages on the basis of phenotype, transcription factor expression and

differentiation pattern (Schulz et al., 2012). Yolk sac-derived macrophages develop independently of c-*Myb* but are dependent on *PU.1*, while bone marrow-derived macrophages are dependent on *c-Myb* but not *PU.*1 (Schulz et al., 2012). An alternative hypothesis for the developmental relationship between L-DC and resident monocytes is that they both derive from a common progenitor in spleen which is yolk sac-derived. However, they would need to diverge at some later point in development, acquiring function and properties reflective of a resident monocyte and a resident DC subset. Further experimental work could investigate the precursor-progeny relationship between these 2 subsets, for example whether one develops from the other, or whether they diverge from a common progenitor.

The possibility that L-DC reflect monocyte-derived DC (mo-DC) was also considered. Mo-DC have been characterised mainly in mouse models of infection. Ly6C$^{hi}$ inflammatory monocytes give rise to mo-DC like TNFα and iNOS-producing DC (Tip-DC) in tissues during inflammation, while resident monocytes give rise to alternative activated M2 macrophages in tissues during the steady-state (Auffray et al., 2007; Auffray et al., 2009). Tip-DC has also been described as classically activated M1 macrophages (Geissmann et al., 2010; Hume, 2012; Serbina et al., 2003). It is notable that L-DC development occurs independently of inflammatory signals (Chapter 7), essential for generation of Tip-DC. In addition, the L-DC phenotype is distinct from Tip-DC by lack of Ly6C and MHCII expression (Chapters 3 and 4), and lack of nitric oxide production (data not shown). These findings show that L-DC are clearly distinct from mo-DC.

Blood resident monocytes have been described as precursors of macrophages in the steady-state (Geissmann et al., 2010), although it is unlikely that they are precursors of yolk sac-derived splenic macrophages since these are thought to develop without a monocytic stage (Takahashi et al., 1989). A contradictory study has shown that splenic macrophages are largely derived from bone marrow-derived monocytes, with none or little contribution from yolk sac-derived progenitors (Ginhoux et al., 2010). In the light of an absence of information on the relationship between splenic macrophages and monocyte subsets, it is impossible at this stage to make predictions about a possible role for the newly defined splenic resident monocyte subset described here. A previous study identified a reservoir of

monocytes in spleen including inflammatory and resident monocytes, both of which were rapidly deployed to sites of inflammation upon induction of myocardial infarction (Swirski et al., 2009). After myocardial infarction, both monocyte subsets exited the spleen and entered the circulation. However, only inflammatory monocytes lodged in heart tissue, while resident monocytes were thought to be distributed into other tissues. That paper identified splenic monocytes as $CD11b^{+}CD11c^{lo}CD115^{+}F4/80^{lo}Ly6G^{-}$ cells which were further delineated on the basis of Ly6C expression (Swirski et al. 2009). According to analyses described here, however, the resident monocyte subset in that study is likely to be contaminated with eosinophils. That study however failed to address the origin of splenic monocytes, whether they were blood monocytes which had lodged in spleen or whether they developed from splenic progenitors.

In terms of the lineage relationship between L-DC and resident monocytes described here, it is possible that these two subsets derive from the same endogenous progenitor in spleen, but acquire specific functions as they develop under the influence of the spleen microenvironment. L-DC could reflect an endogenous DC population with specific function in cross-presentation of blood-borne antigen to $CD8^{+}$ T cells, while splenic resident monocytes could reflect a population of monocytes specialised in clearance of blood-borne antigens evidenced by their ability to endocytose both soluble and particulate molecules.

## 8.5 Relationship between inflammatory monocytes and resident monocytes

Blood monocytes develop from a common myeloid progenitor (CMP) in bone marrow and enter circulation as mature cells. Recently, an immediate precursor of monocytes, the common monocyte progenitor (CMoP) was also described in blood (Hettinger et al., 2013). CMoP were found to give rise to inflammatory and resident monocytes, and to macrophages following macrophage deletion or inflammation, but they did not give rise to DC (Hettinger et al., 2013). While that study has defined an important progenitor subset, it remains to be investigated whether spleen resident monocytes and inflammatory monocytes described here arise directly from CMoP.

The resident monocyte and inflammatory monocyte subsets identified here in spleen appear to be closely related in terms of gene expression (Chapter 6). Clustering analysis has shown that both subsets share similar gene expression profiles, although they are not as closely related as are resident monocytes with L-DC subset. Comparison between L-DC, inflammatory monocytes and resident monocytes led to the identification of *Fn1*, *F13a1* and *Mmp8* as genes encoding specific markers of inflammatory monocytes. Inflammatory monocytes were also distinguished from resident monocytes by upregulated expression of *Mmp8*, *F13a1*, *Vcan*, *Cd14* and *Fn1*, while resident monocytes were characterised by upregulation of *Ccl5*, *Dusp16*, *Cd36*, *H2-Ab1* and *Fabp4* genes.

Both monocyte subsets appear to develop independently. Inflammatory monocytes but not resident monocytes depend on FLT3L and GM-CSF for development in spleen. Resident monocytes are dependent on TLR3 and TLR4 signalling, while inflammatory monocyte develop in the absence of inflammation and are dependent on the BATF3 and C-MYB transcription factors. Further investigation of the lineage origin of resident monocytes and inflammatory monocytes, and their relation to L-DC lineage will be essential for further understanding their functional role in spleen.

## 8.6 Conclusions and future work

This study has identified three specific markers which could be potential identifiers of the L-DC subset in spleen. These include CD300E, CD300LD and CD9. Available antibodies for CD300E and CD300LD are however specific for human and not murine molecules, and did not show staining of murine splenocytes (data not shown). Further experimental work will be dependent on production of antibodies specific for the murine CD300E and CD300LD molecules. A *CD300e*$^{-/-}$ knockout mouse strain has been developed by Georgina Clark (University of Sydney, NSW, Australia) which will also be important in further studies in order to assess the development and function of L-DC in murine spleen. Lastly, it will be important to translate this murine study into the human model, and to identify a human counterpart of L-DC, and to establish human splenic longterm cultures which produce LTC-DC.

In conclusion, L-DC represent a novel subset of antigen presenting cells that have not previously been described in spleen. They have distinct potential in cross-presentation and could play an important role in immunity to blood-borne infections and to cancer. In addition, the availability of a culture system that generates large numbers of cells equivalent to L-DC could have potential therapeutic value in personalised medicine.

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# Appendices

**Figure A.1 Identification of eosinophil phenotype.**

Spleen cells were prepared by red blood cell lysis followed by T and B cell depletion, and then stained with antibodies specific for CD11b (M1/70, PE-Cy7), CD11c (N418, APC), Ly6C (AL-21, Pacific Blue), Ly6G (1A8, Bio), CD43 (IBII, Alexa488) and Siglec-F (E50-2440, PE), using streptavidin (SA)-APC-Cy7 as a second stage reagent. Prior to flow cytometry, cells were stained with propidium iodide (PI, 1μg/ml) to delineate live ($PI^-$) cells. Siglec-$F^+$ cells were gated and further delineated on the basis of CD11b, CD11c, Ly6C, Ly6G and CD43 expression. The majority of the Siglec-$F^+$ cells expressed the phenotype of $CD11b^{hi}Ly6C^+CD43^{hi}$ cells. Gates were set based on fluorescence minus one controls, and numbers in gates represent % specific binding.

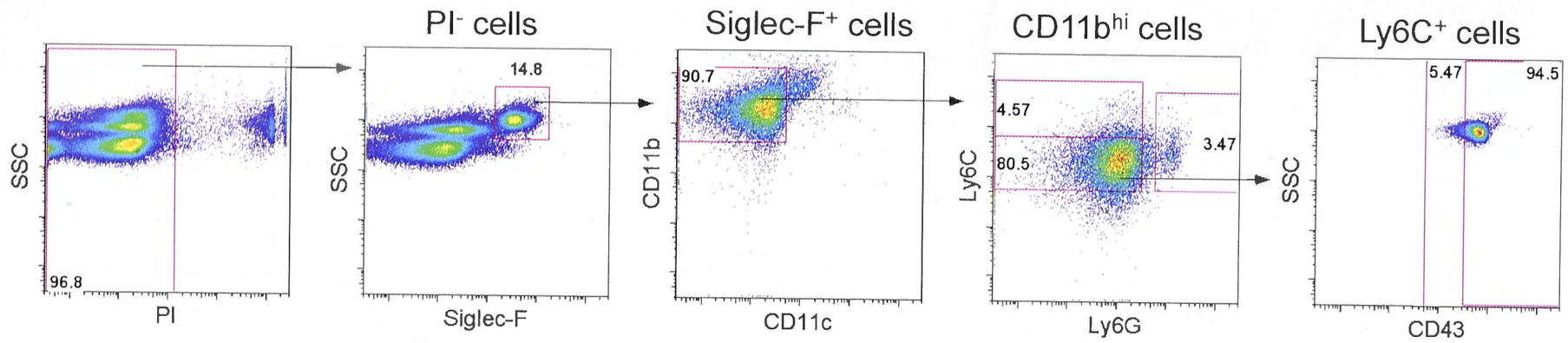
PI⁻ cells
Siglec-F⁺ cells
CD11b^hi cells
Ly6C⁺ cells
SSC
PI
96.8
14.8
SSC
Siglec-F
CD11b
CD11c
90.7
Ly6C
Ly6G
4.57
80.5
3.47
SSC
CD43
5.47
94.5

**Figure A.2 Expression of Ly6C and Ly6G on L-DC candidates.**

Splenocytes were prepared and depleted of T and B cells as described in the legend to Figure 3.2. Cells were then stained with antibodies specific for CD11b (M1/70, PE-Cy7), CD11c (N418, APC), MHCII (25-9-17, Biotin), Ly6C (Al-21, FITC) and Ly6G (1A8, PE), along with propidium iodide (PI, 1μg/ml) for gating live ($PI^-$) cells. L-DC were gated as $CD11b^{hi}CD11c^{lo}MHCII^-$ cells, then delineated on the basis of SSC and Ly6G expression. Three candidate L-DC subsets have been identified as $SSC^{lo}Ly6G^-$, $SSC^{hi}Ly6G^-$ and $SSC^{mid}Ly6G^+$ cells. Expression of Ly6C on these subsets was identified. Gates were set based on fluorescence minus one controls and numbers in gates represent % specific gating.

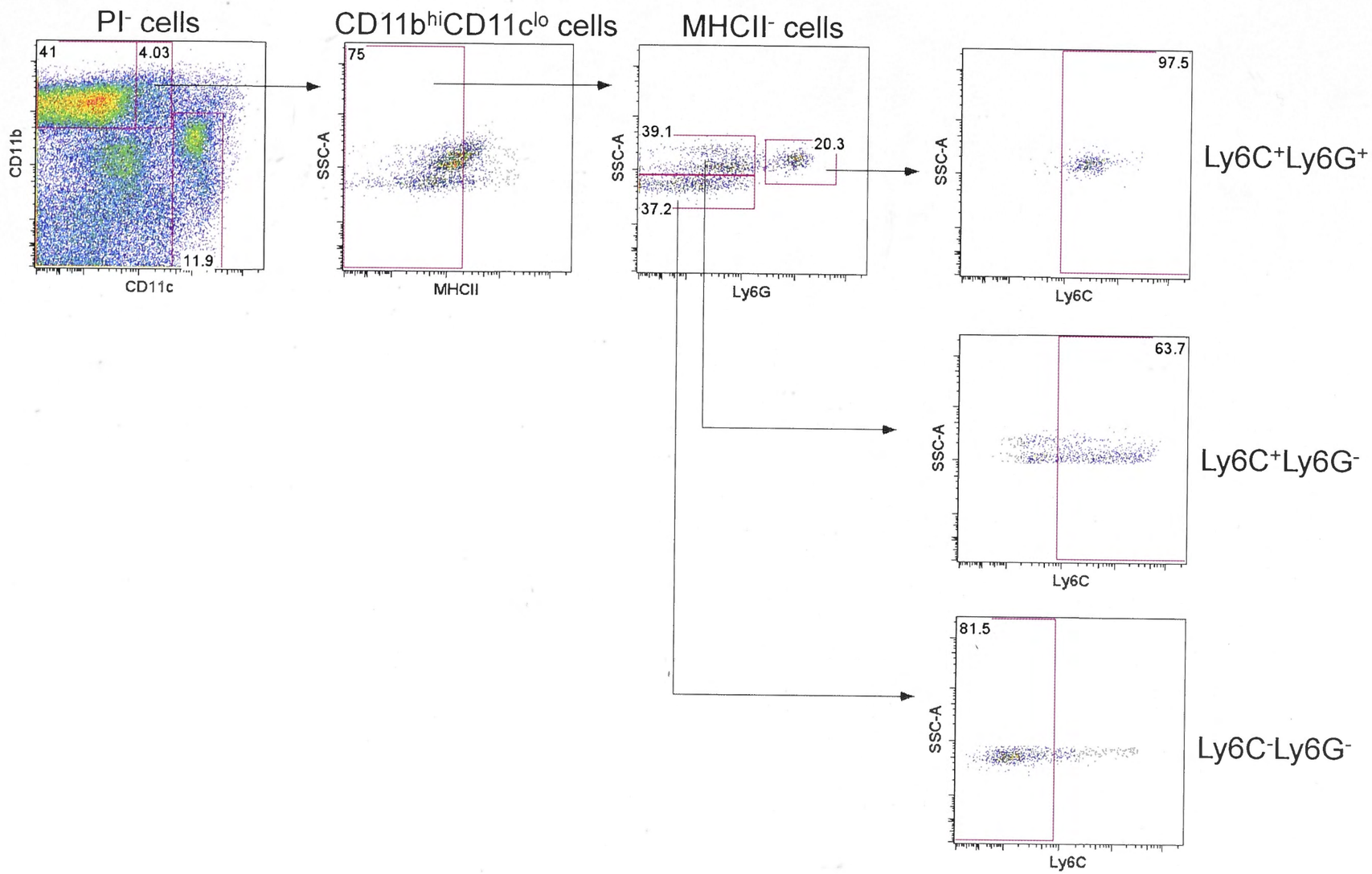
PI- cells
CD11bhiCD11clo cells
MHCII- cells
41
4.03
11.9
CD11b
CD11c
75
SSC-A
MHCII
39.1
20.3
37.2
SSC-A
Ly6G
97.5
SSC-A
Ly6C
Ly6C+Ly6G+
63.7
SSC-A
Ly6C
Ly6C+Ly6G-
81.5
SSC-A
Ly6C
Ly6C-Ly6G-

**Figure A.3 Relationship between sorted dendritic and myeloid subsets and tissue macrophages.**

A set of genes uniquely expressed by tissue macrophages was identified by Gautier et al. (2012). The expression of these genes is shown for each of the L-DC, cDC and myeloid subsets. For each of the subsets, signal values were plotted as a heat map using R/Bioconductor software. The line chart (blue) overlaid on heat maps indicates signal intensity changes about the mean (dashed blue line).

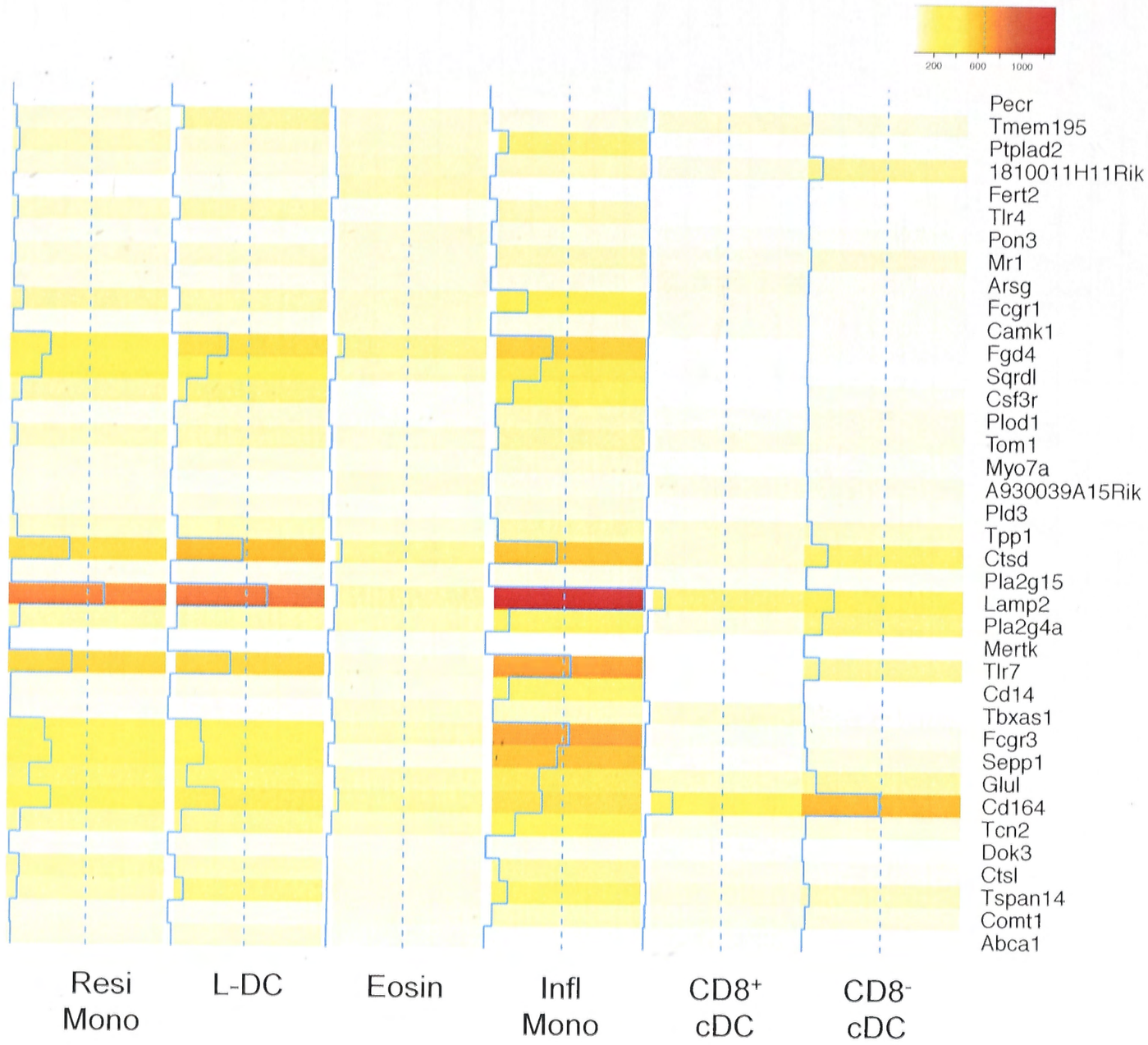
200
600
1000
Pecr
Tmem195
Ptplad2
1810011H11Rik
Fert2
Tlr4
Pon3
Mr1
Arsg
Fcgr1
Camk1
Fgd4
Sqrdl
Csf3r
Plod1
Tom1
Myo7a
A930039A15Rik
Pld3
Tpp1
Ctsd
Pla2g15
Lamp2
Pla2g4a
Mertk
Tlr7
Cd14
Tbxas1
Fcgr3
Sepp1
Glul
Cd164
Tcn2
Dok3
Ctsl
Tspan14
Comt1
Abca1
Resi Mono
L-DC
Eosin
Infl Mono
CD8+ cDC
CD8- cDC

**Table A.1 Genes upregulated between $CD8^+$ cDC and $CD8^-$ cDC.**

| Gene Symbol | Fold-Change ($CD8^+$ cDC vs. $CD8^-$ cDC) [+] | $CD8^+$ cDC | | $CD8^-$ cDC | |
|---|---|---|---|---|---|
| | | Mean* | Standard Error | Mean* | Standard Error |
| *Cd8a* | 27.760 | 2195.601 | 1.674 | 80.206 | 13.316 |
| *Leprel1* | 18.700 | 980.770 | 41.569 | 52.682 | 5.428 |
| *Clec9a* | 11.996 | 3392.192 | 5.644 | 285.108 | 36.356 |
| *F630111L10Rik* | 11.788 | 631.032 | 28.134 | 53.497 | 1.357 |
| *Xcr1* | 10.811 | 1868.014 | 75.846 | 172.892 | 9.038 |
| *Cxcl9* | 8.523 | 846.279 | 6.207 | 99.470 | 5.929 |
| *Cadm1* | 8.389 | 649.668 | 6.609 | 77.474 | 2.497 |
| *Cd36* | 8.135 | 1277.064 | 30.626 | 158.133 | 19.431 |
| *A530099J19Rik* | 7.745 | 2044.525 | 61.354 | 266.319 | 36.026 |
| *Ppap2a* | 7.671 | 394.075 | 20.952 | 51.451 | 3.940 |
| *5430435G22Rik* | 7.332 | 714.641 | 55.541 | 97.175 | 0.063 |
| *Irf8* | 7.213 | 520.587 | 61.997 | 71.740 | 3.470 |
| *Gclc* | 6.979 | 839.291 | 69.400 | 120.956 | 16.279 |
| *Zc3h12c* | 6.957 | 629.493 | 17.648 | 91.751 | 15.419 |
| *P2ry14* | 6.883 | 722.152 | 27.864 | 105.120 | 7.736 |
| *Pdia5* | 6.205 | 360.117 | 40.376 | 57.807 | 3.982 |
| *Serpinb6b* | 6.093 | 757.433 | 28.334 | 125.936 | 20.695 |
| *Tlr11* | 5.970 | 866.195 | 13.192 | 147.310 | 25.528 |
| *Zc3h12c* | 5.856 | 539.440 | 8.477 | 93.220 | 14.333 |
| *Cacna1e* | 5.843 | 461.562 | 27.567 | 78.868 | 1.165 |
| *Ly75* | 5.827 | 603.775 | 10.825 | 103.809 | 6.585 |
| *Mpeg1* | 5.729 | 1856.619 | 31.853 | 324.460 | 17.125 |
| *Naaa* | 5.448 | 1520.239 | 34.984 | 279.251 | 12.111 |
| *Tlr3* | 5.431 | 452.356 | 2.728 | 83.351 | 3.261 |
| *Slc24a6* | 5.343 | 357.848 | 21.183 | 66.928 | 3.052 |
| *Pygl* | -5.007 | 51.034 | 11.070 | 252.082 | 36.260 |
| *Tlr1* | -5.011 | 145.418 | 10.188 | 727.615 | 32.956 |
| *Ffar2* | -5.066 | 51.473 | 3.337 | 261.555 | 26.392 |
| *Ccrl2* | -5.117 | 76.939 | 4.528 | 394.462 | 33.476 |
| *Ap1s2* | -5.593 | 56.170 | 1.089 | 315.051 | 24.636 |
| *Ms4a6c* | -5.642 | 156.529 | 21.362 | 875.087 | 17.948 |
| *Man1a* | -6.100 | 91.439 | 1.285 | 559.336 | 42.041 |
| *B4galt6* | -6.126 | 54.736 | 7.457 | 333.803 | 32.710 |
| *Klri2* | -6.488 | 52.744 | 0.753 | 346.550 | 54.901 |
| *Lair1* | -6.836 | 80.834 | 7.688 | 558.137 | 94.371 |
| *Fgr* | -6.849 | 62.017 | 0.315 | 427.433 | 47.658 |
| *St8sia6* | -6.876 | 56.681 | 4.728 | 391.098 | 46.106 |
| *Cyp4f16* | -7.390 | 139.444 | 8.088 | 1028.779 | 9.949 |
| *Cd300lf* | -7.466 | 58.307 | 7.648 | 431.599 | 2.847 |
| *Abcb1a* | -7.508 | 54.137 | 9.155 | 401.695 | 29.625 |
| *Pgap1* | -7.552 | 82.519 | 0.523 | 628.003 | 78.002 |
| *Fcer1g* | -7.734 | 122.952 | 0.040 | 951.812 | 41.738 |
| *Cybb* | -7.785 | 240.468 | 1.650 | 1872.053 | 5.191 |
| *Ptgs2* | -7.804 | 111.339 | 3.020 | 868.571 | 0.638 |
| *Cd209a* | -7.915 | 90.135 | 15.468 | 706.500 | 71.983 |
| *Gpr141* | -7.930 | 51.999 | 2.702 | 411.941 | 10.842 |
| *Sirpa* | -7.943 | 104.930 | 0.800 | 833.676 | 18.321 |
| *Ccl3* | -8.048 | 184.994 | 4.515 | 1488.726 | 31.009 |
| *Rgl1* | -8.109 | 68.415 | 4.020 | 555.152 | 38.649 |
| *Ms4a6b* | -8.132 | 83.506 | 4.574 | 678.064 | 4.506 |
| *Arap2* | -8.267 | 81.273 | 5.637 | 671.226 | 36.532 |
| *Gm1966* | -8.405 | 132.006 | 9.473 | 1109.452 | 79.698 |
| *Klrd1* | -8.650 | 166.452 | 28.536 | 1418.506 | 4.376 |
| *Cd7* | -8.708 | 120.259 | 15.711 | 1039.292 | 46.182 |

| | | | | | |
|---|---|---|---|---|---|
| *S100a4* | -9.890 | 95.820 | 14.184 | 940.997 | 84.081 |
| *Mmp12* | -11.009 | 50.726 | 1.200 | 559.861 | 41.845 |
| *Ms4a4c* | -12.251 | 79.293 | 8.637 | 965.636 | 0.177 |
| *Emb* | -14.906 | 50.480 | 6.721 | 747.515 | 51.220 |
| *Sirpb1b* | -14.911 | 76.957 | 7.361 | 1142.435 | 21.104 |
| *Sirpb1a* | -15.369 | 107.658 | 12.318 | 1643.742 | 8.262 |
| *Sirpb1a* | -16.575 | 91.904 | 10.731 | 1513.692 | 48.988 |
| *Sirpb1b* | -17.607 | 89.728 | 10.088 | 1570.473 | 46.530 |

[+] Genes were selected which showed ≥ 5 fold change in signal value in either $CD8^+$ cDC or $CD8^-$ cDC assessed in pairwise comparison.

* Data represent mean of duplicate samples (n=2).

## Table A.3 Genes upregulated between L-DC and CD8$^-$ cDC.

| Gene Symbol | Fold-Change (L-DC vs. CD8$^-$ cDC) $^+$ | L-DC | | CD8$^-$ cDC | |
|---|---|---|---|---|---|
| | | Mean* | Standard Error | Mean* | Standard Error |
| *Klra2* | 26.329 | 1450.602 | 110.356 | 56.154 | 11.631 |
| *Pilra* | 23.659 | 1160.911 | 58.620 | 50.588 | 12.555 |
| *Cd300e* | 16.170 | 1421.362 | 12.809 | 88.025 | 4.742 |
| *Ceacam1* | 14.725 | 1321.885 | 70.953 | 89.675 | 2.437 |
| *Fcgr4* | 14.092 | 1084.527 | 0.263 | 76.960 | 0.015 |
| *Ace* | 13.357 | 989.627 | 73.881 | 73.984 | 3.797 |
| *Emr4* | 12.281 | 2439.648 | 139.380 | 198.545 | 9.187 |
| *Cd36* | 11.903 | 1881.042 | 220.923 | 158.133 | 19.431 |
| *Cd300ld* | 11.635 | 1120.164 | 83.248 | 96.918 | 13.244 |
| *Cpd* | 10.867 | 548.903 | 12.224 | 50.622 | 3.543 |
| *Pilrb1* | 9.652 | 806.360 | 60.236 | 83.536 | 6.164 |
| *Rasa3* | 9.302 | 491.216 | 18.542 | 53.035 | 5.284 |
| *C3* | 8.317 | 425.896 | 13.786 | 51.240 | 2.417 |
| *Nupr1* | 7.538 | 481.366 | 36.486 | 63.685 | 1.325 |
| *Slc11a1* | 7.523 | 389.184 | 9.197 | 51.735 | 1.265 |
| *Smpdl3a* | 7.471 | 1163.378 | 90.269 | 155.623 | 10.940 |
| *Csf1r* | 7.388 | 1335.560 | 100.913 | 181.666 | 22.470 |
| *Ly6i* | 7.330 | 414.900 | 43.280 | 57.882 | 13.457 |
| *Ccl6* | 7.267 | 743.856 | 8.929 | 102.348 | 0.479 |
| *Grk5* | 7.062 | 417.621 | 13.741 | 59.721 | 8.576 |
| *Plin2* | 7.040 | 819.562 | 65.949 | 117.109 | 15.807 |
| *Cx3cr1* | 6.947 | 637.558 | 46.178 | 92.441 | 12.915 |
| *L1cam* | 6.882 | 448.746 | 22.570 | 65.447 | 6.506 |
| *Gsr* | 6.844 | 659.658 | 44.670 | 96.472 | 7.621 |
| *E430024C06Rik* | 6.758 | 1568.881 | 44.092 | 232.069 | 3.002 |
| *Trem3* | 6.660 | 357.258 | 13.473 | 53.603 | 0.252 |
| *Hpgd* | 6.636 | 2346.482 | 46.518 | 354.083 | 19.447 |
| *Itgal* | 6.626 | 1244.192 | 117.344 | 187.209 | 10.115 |
| *Cfh* | 6.528 | 559.477 | 20.458 | 85.863 | 6.024 |
| *Apoe* | 6.504 | 526.683 | 49.335 | 80.640 | 1.650 |
| *Heg1* | 6.404 | 466.517 | 9.132 | 72.966 | 4.383 |
| *Treml4* | 6.357 | 2158.616 | 126.754 | 339.569 | 19.967 |
| *Lrp1* | 6.310 | 375.113 | 16.648 | 59.407 | 1.334 |
| *Ldlrad3* | 6.141 | 388.563 | 14.701 | 63.290 | 2.838 |
| *Tmem38b* | 6.095 | 369.920 | 13.911 | 60.656 | 0.946 |
| *Spn* | 5.967 | 536.108 | 0.126 | 89.851 | 0.915 |
| *Gm11711* | 5.951 | 939.789 | 148.111 | 156.649 | 14.680 |
| *Clec4a3* | 5.852 | 2663.629 | 105.292 | 458.377 | 56.846 |
| *Gm11711* | 5.793 | 862.056 | 168.664 | 146.209 | 9.082 |
| *Gzmb* | 5.755 | 451.871 | 24.728 | 80.416 | 17.915 |
| *Ikzf3* | 5.732 | 290.713 | 35.031 | 50.415 | 2.588 |
| *Mpp1* | 5.724 | 545.943 | 83.878 | 95.434 | 15.059 |
| *Dusp16* | 5.715 | 877.419 | 64.334 | 153.858 | 15.115 |
| *Sepx1* | 5.638 | 296.277 | 6.127 | 52.605 | 2.590 |
| *Rras* | 5.559 | 496.804 | 51.089 | 88.937 | 2.818 |
| *Idh1* | 5.559 | 301.556 | 55.509 | 53.422 | 3.349 |
| *Rbpms* | 5.465 | 288.010 | 12.871 | 52.652 | 0.388 |
| *Sepp1* | 5.448 | 326.572 | 47.610 | 59.464 | 4.316 |
| *S1pr5* | 5.352 | 303.395 | 4.288 | 56.686 | 0.514 |
| *Cyth3* | 5.276 | 309.885 | 27.349 | 58.531 | 1.589 |
| *Atp1a3* | 5.008 | 528.196 | 5.192 | 105.459 | 0.591 |
| *Rogdi* | -5.094 | 50.934 | 1.188 | 259.663 | 11.643 |
| *Top2a* | -5.111 | 88.695 | 3.295 | 456.700 | 57.831 |
| *Egr2* | -5.111 | 56.160 | 4.636 | 286.071 | 2.981 |

| | | | | | |
|---|---|---|---|---|---|
| *Anxa3* | -5.111 | 78.507 | 1.400 | 403.640 | 44.751 |
| *Cd22* | -5.117 | 62.146 | 2.741 | 318.066 | 15.864 |
| *Tspan2* | -5.125 | 64.412 | 3.474 | 330.478 | 24.040 |
| *Gpr183* | -5.129 | 96.407 | 1.247 | 494.547 | 9.969 |
| *Slc44a1* | -5.151 | 158.358 | 6.449 | 815.594 | 29.149 |
| *Cd74* | -5.174 | 495.887 | 33.751 | 2561.664 | 102.945 |
| *Cd300lf* | -5.206 | 83.051 | 5.041 | 431.599 | 2.847 |
| *P2ry10* | -5.238 | 87.687 | 5.633 | 459.248 | 29.021 |
| *H2-Ab1* | -5.530 | 673.974 | 30.767 | 3723.520 | 53.946 |
| *Kif11* | -5.547 | 50.132 | 0.942 | 278.399 | 14.182 |
| *Mdh2* | -5.847 | 331.933 | 14.718 | 1940.908 | 87.468 |
| *Ifi205* | -5.983 | 112.100 | 11.276 | 667.351 | 10.170 |
| *Abcb1a* | -6.208 | 64.531 | 0.865 | 401.695 | 29.625 |
| *Cd7* | -6.403 | 162.687 | 13.288 | 1039.292 | 46.182 |
| *Cd80* | -6.417 | 54.467 | 1.371 | 350.752 | 30.798 |
| *Dpp4* | -6.429 | 197.603 | 16.634 | 1265.903 | 10.619 |
| *Chn2* | -6.465 | 59.894 | 1.464 | 388.241 | 30.106 |
| *H2-Eb1* | -6.591 | 331.002 | 10.861 | 2182.665 | 100.326 |
| *Tlr1* | -6.706 | 108.388 | 0.201 | 727.615 | 32.956 |
| *Tfrc* | -7.028 | 67.921 | 4.917 | 477.326 | 34.672 |
| *Nedd4* | -7.484 | 72.524 | 0.172 | 543.586 | 30.297 |
| *Rgs1* | -7.876 | 337.758 | 42.980 | 2638.609 | 10.701 |
| *Apol7c* | -8.206 | 129.958 | 51.582 | 979.190 | 26.671 |
| *Arap2* | -8.283 | 80.973 | 2.949 | 671.226 | 36.532 |
| *Kynu* | -8.337 | 129.013 | 17.380 | 1066.387 | 35.841 |
| *Rgl1* | -8.460 | 65.465 | 0.460 | 555.152 | 38.649 |
| *Rras2* | -8.852 | 86.797 | 0.272 | 768.375 | 9.774 |
| *Flt3* | -9.274 | 107.580 | 12.807 | 990.702 | 16.190 |
| *Rnd3* | -9.561 | 99.003 | 8.929 | 945.627 | 73.852 |
| *Clec4a4* | -9.672 | 63.153 | 5.598 | 610.519 | 50.632 |
| *Plxnc1* | -10.177 | 146.779 | 1.462 | 1493.675 | 12.529 |
| *Mmp12* | -10.510 | 53.148 | 1.701 | 559.861 | 41.845 |
| *Klri1* | -12.391 | 60.717 | 1.657 | 752.187 | 13.788 |
| *Ptgs2* | -12.623 | 69.661 | 10.852 | 868.571 | 0.638 |
| *Ccnd1* | -12.824 | 70.079 | 2.545 | 899.306 | 46.231 |
| *H2-Ob* | -15.387 | 65.471 | 3.630 | 1008.020 | 66.006 |
| *H2-DMb2* | -16.814 | 67.368 | 3.166 | 1132.532 | 49.628 |
| *Dnase1l3* | -18.035 | 56.513 | 3.214 | 1018.803 | 49.799 |
| *Adam23* | -24.250 | 62.837 | 5.703 | 1517.628 | 18.095 |

[+] Genes were selected which showed ≥ 5 fold change in signal value in either L-DC or $CD8^-$ cDC assessed in pairwise comparison.

* Data represent mean of duplicate samples (n=2).

**Table A.2 Genes upregulated between L-DC and $CD8^+$ cDC.**

| Gene Symbol | Fold-Change (L-DC vs. $CD8^+$ cDC) $^+$ | L-DC | | $CD8^+$ cDC | |
|---|---|---|---|---|---|
| | | Mean* | Standard Error | Mean* | Standard Error |
| *Sirpb1b* | 20.229 | 1817.123 | 220.432 | 89.728 | 10.088 |
| *Hpgd* | 18.954 | 2346.482 | 46.518 | 123.921 | 6.043 |
| *Cybb* | 18.442 | 4461.392 | 487.951 | 240.468 | 1.650 |
| *Ear2* | 18.372 | 3381.838 | 125.371 | 183.967 | 2.385 |
| *Pglyrp1* | 17.279 | 1049.278 | 117.991 | 60.495 | 4.309 |
| *Emb* | 16.666 | 842.257 | 118.940 | 50.480 | 6.721 |
| *Sirpb1b* | 16.652 | 1283.321 | 140.667 | 76.957 | 7.361 |
| *Lair1* | 16.436 | 1322.610 | 7.289 | 80.834 | 7.688 |
| *Cd300e* | 15.779 | 1421.362 | 12.809 | 90.094 | 1.662 |
| *Fcer1g* | 15.199 | 1872.824 | 122.647 | 122.952 | 0.040 |
| *Sirpb1a* | 14.649 | 1573.238 | 142.862 | 107.658 | 12.318 |
| *Fgr* | 14.323 | 888.436 | 18.826 | 62.017 | 0.315 |
| *Sirpb1a* | 14.278 | 1304.114 | 48.662 | 91.904 | 10.731 |
| *Gngt2* | 14.107 | 718.670 | 31.025 | 50.928 | 1.766 |
| *Ear10* | 13.892 | 1884.006 | 46.422 | 135.584 | 1.707 |
| *Stk10* | 13.758 | 817.522 | 63.934 | 59.738 | 7.702 |
| *Ear1* | 13.525 | 1465.111 | 47.061 | 108.343 | 3.871 |
| *Pygl* | 13.078 | 651.837 | 19.608 | 51.034 | 11.070 |
| *Gpr141* | 12.734 | 663.209 | 51.012 | 51.999 | 2.702 |
| *Ap1s2* | 12.508 | 702.750 | 20.242 | 56.170 | 1.089 |
| *Cx3cr1* | 11.532 | 637.558 | 46.178 | 55.147 | 0.959 |
| *Ear10* | 10.956 | 1306.305 | 31.735 | 119.219 | 2.629 |
| *Ctsd* | 10.442 | 627.410 | 12.664 | 60.518 | 7.339 |
| *Klra10* | 10.156 | 619.711 | 4.363 | 61.109 | 3.378 |
| *Klra9* | 9.815 | 521.935 | 6.317 | 53.229 | 2.408 |
| *Serpinb10-ps* | 9.815 | 1174.014 | 240.742 | 117.333 | 7.893 |
| *Gm11711* | 9.746 | 939.789 | 148.111 | 95.224 | 0.298 |
| *Ssh2* | 9.490 | 1615.582 | 73.366 | 170.142 | 5.252 |
| *Gm11711* | 9.219 | 862.056 | 168.664 | 91.700 | 0.796 |
| *Ccl3* | 8.631 | 1598.800 | 92.611 | 184.994 | 4.515 |
| *Ccl6* | 8.493 | 743.856 | 8.929 | 87.586 | 1.229 |
| *Ncf2* | 8.445 | 451.472 | 9.861 | 53.477 | 1.683 |
| *Mpp1* | 8.226 | 545.943 | 83.878 | 65.610 | 2.027 |
| *Smpdl3a* | 8.164 | 1163.378 | 90.269 | 144.203 | 24.696 |
| *Dram2* | 7.986 | 619.554 | 82.119 | 77.477 | 9.491 |
| *Slc12a2* | 7.979 | 1866.628 | 281.744 | 231.534 | 11.198 |
| *Ms4a6b* | 7.788 | 650.514 | 38.315 | 83.506 | 4.574 |
| *Sirpa* | 7.760 | 815.666 | 47.589 | 104.930 | 0.800 |
| *Ly6i* | 7.691 | 414.900 | 43.280 | 53.701 | 2.291 |
| *Stap1* | 7.678 | 2172.126 | 78.495 | 283.002 | 12.806 |
| *Spn* | 7.674 | 536.108 | 0.126 | 69.880 | 1.582 |
| *Ms4a6c* | 7.531 | 1168.381 | 36.276 | 156.529 | 21.362 |
| *Gm1966* | 7.475 | 984.371 | 18.079 | 132.006 | 9.473 |
| *Ptpn12* | 7.430 | 1341.571 | 16.368 | 180.944 | 12.069 |
| *Ifitm3* | 7.059 | 5280.975 | 38.623 | 748.077 | 3.257 |
| *Fam111a* | 7.011 | 741.106 | 22.347 | 106.131 | 9.938 |
| *Add3* | 6.853 | 522.108 | 54.546 | 76.290 | 8.863 |
| *Ifitm2* | 6.765 | 864.336 | 9.847 | 127.846 | 4.588 |
| *Ldlrad3* | 6.709 | 388.563 | 14.701 | 57.881 | 0.633 |
| *Cfh* | 6.613 | 559.477 | 20.458 | 84.722 | 5.395 |
| *E430024C06Rik* | 6.553 | 1568.881 | 44.092 | 239.414 | 7.163 |
| *E430024C06Rik* | 6.553 | 1568.881 | 44.092 | 239.414 | 7.163 |
| *Ms4a4c* | 6.463 | 509.466 | 1.648 | 79.293 | 8.637 |
| *G6pdx* | 6.457 | 669.443 | 46.765 | 103.935 | 10.276 |

| | | | | | |
|---|---|---|---|---|---|
| *Itgal* | 6.451 | 1244.192 | 117.344 | 194.106 | 28.443 |
| *Tnfrsf1b* | 6.363 | 729.939 | 17.751 | 114.757 | 4.333 |
| *Lyz2* | 6.336 | 5511.231 | 372.900 | 868.677 | 38.348 |
| *Lpcat2* | 6.202 | 462.187 | 27.825 | 74.585 | 5.423 |
| *Rras* | 6.152 | 496.804 | 51.089 | 80.552 | 6.033 |
| *Il17ra* | 6.137 | 442.363 | 32.134 | 72.008 | 4.190 |
| *Sepx1* | 5.946 | 296.277 | 6.127 | 50.268 | 6.690 |
| *Tmem38b* | 5.909 | 369.920 | 13.911 | 62.620 | 2.688 |
| *Cd300a* | 5.875 | 1186.579 | 36.758 | 202.246 | 12.185 |
| *Msn* | 5.764 | 2989.141 | 245.111 | 517.242 | 20.514 |
| *Nadk* | 5.747 | 649.019 | 39.027 | 114.709 | 21.258 |
| *Dusp16* | 5.740 | 877.419 | 64.334 | 153.463 | 17.710 |
| *Nfe2l2* | 5.677 | 1293.088 | 222.153 | 228.891 | 45.185 |
| *Ugcg* | 5.671 | 608.982 | 10.292 | 108.164 | 13.079 |
| *Nr4a1* | 5.665 | 989.626 | 0.254 | 175.483 | 16.710 |
| *Casp4* | 5.655 | 389.013 | 24.212 | 69.542 | 11.092 |
| *Bin2* | 5.651 | 354.086 | 38.551 | 62.357 | 2.946 |
| *Slc44a2* | 5.623 | 632.895 | 24.218 | 113.100 | 11.936 |
| *Gsr* | 5.594 | 659.658 | 44.670 | 117.690 | 2.687 |
| *Klri2* | 5.592 | 295.497 | 18.183 | 52.744 | 0.753 |
| *Ifitm1* | 5.580 | 432.218 | 17.171 | 77.397 | 0.617 |
| *Casp1* | 5.511 | 599.420 | 74.556 | 107.938 | 2.119 |
| *Tgfbr1* | 5.474 | 710.148 | 36.679 | 129.721 | 6.712 |
| *Ifitm1* | 5.465 | 283.918 | 19.475 | 52.051 | 4.798 |
| *Acot9* | 5.452 | 484.332 | 14.049 | 88.851 | 2.988 |
| *Emp3* | 5.418 | 607.557 | 88.653 | 111.639 | 12.422 |
| *Cpd* | 5.360 | 548.903 | 12.224 | 103.941 | 17.956 |
| *Sh3kbp1* | 5.353 | 702.446 | 2.364 | 131.409 | 7.059 |
| *Gm885* | 5.314 | 324.725 | 21.441 | 61.164 | 4.845 |
| *Smc6* | 5.312 | 1014.524 | 80.477 | 190.495 | 6.000 |
| *Mettl7a1* | 5.192 | 318.346 | 11.740 | 61.410 | 4.075 |
| *Eno1* | 5.174 | 435.383 | 7.718 | 84.935 | 11.634 |
| *Klra3* | 5.109 | 375.137 | 6.290 | 73.500 | 3.450 |
| *Igsf6* | 5.062 | 1790.305 | 71.141 | 353.625 | 12.118 |
| *Rasa3* | 5.037 | 491.216 | 18.542 | 97.564 | 4.592 |
| *Apoe* | 5.021 | 526.683 | 49.335 | 104.436 | 0.645 |
| *Mlec* | -5.022 | 75.511 | 8.497 | 379.691 | 46.827 |
| *Slamf8* | -5.093 | 54.877 | 2.822 | 279.847 | 20.152 |
| *Klri1* | -5.155 | 60.717 | 1.657 | 312.952 | 7.292 |
| *Cd81* | -5.176 | 166.837 | 9.362 | 871.281 | 125.849 |
| *Etv3* | -5.178 | 66.731 | 3.967 | 344.928 | 2.060 |
| *Tspan3* | -5.210 | 103.509 | 3.667 | 539.128 | 13.661 |
| *Dpp4* | -5.319 | 197.603 | 16.634 | 1047.465 | 14.413 |
| *Slco3a1* | -5.345 | 59.599 | 7.297 | 316.613 | 16.614 |
| *Cd74* | -5.408 | 495.887 | 33.751 | 2686.703 | 244.990 |
| *A430084P05Rik* | -5.491 | 67.071 | 10.032 | 364.384 | 13.358 |
| *Cpne2* | -5.535 | 63.129 | 4.825 | 350.438 | 37.882 |
| *Strbp* | -5.540 | 71.880 | 5.453 | 398.445 | 33.160 |
| *Gclc* | -5.587 | 150.240 | 12.684 | 839.291 | 69.400 |
| *Myo9a* | -5.611 | 78.107 | 8.604 | 435.580 | 2.807 |
| *Tbc1d9* | -5.724 | 83.569 | 4.165 | 477.986 | 13.879 |
| *Myo9a* | -5.774 | 137.488 | 22.361 | 791.678 | 114.617 |
| *Lrrc18* | -5.845 | 60.960 | 5.492 | 355.076 | 12.291 |
| *Ppt1* | -5.853 | 634.664 | 70.073 | 3694.216 | 119.302 |
| *Fnbp1* | -5.856 | 191.828 | 2.490 | 1127.571 | 98.748 |
| *Pak1* | -5.861 | 103.911 | 1.060 | 610.027 | 34.972 |
| *Slc9a9* | -5.868 | 154.842 | 1.085 | 908.673 | 15.612 |
| *Pik3cb* | -5.901 | 120.383 | 8.550 | 712.350 | 73.358 |
| *H2-Ab1* | -6.001 | 673.974 | 30.767 | 4051.319 | 296.617 |
| *Gramd3* | -6.005 | 56.670 | 8.137 | 336.807 | 3.139 |

| | | | | | |
|---|---|---|---|---|---|
| *Zc3h12c* | -6.184 | 87.452 | 6.271 | 539.440 | 8.477 |
| *Myo9a* | -6.330 | 116.261 | 15.350 | 730.233 | 32.075 |
| *H2-Eb1* | -6.390 | 331.002 | 10.861 | 2116.498 | 106.068 |
| *Atpif1* | -6.468 | 170.249 | 5.469 | 1100.543 | 0.305 |
| *Anxa3* | -6.580 | 78.507 | 1.400 | 516.923 | 20.808 |
| *Myo9a* | -6.658 | 91.101 | 12.687 | 600.683 | 5.719 |
| *Zfp366* | -6.796 | 54.359 | 3.008 | 368.882 | 5.398 |
| *Wdfy4* | -6.940 | 170.770 | 22.100 | 1175.192 | 7.617 |
| *Ppap2a* | -7.093 | 55.491 | 1.091 | 394.075 | 20.952 |
| *Nedd4* | -7.188 | 72.524 | 0.172 | 521.334 | 1.146 |
| *Map2k6* | -7.200 | 54.172 | 5.738 | 388.136 | 15.413 |
| *Irf8* | -7.350 | 70.327 | 0.248 | 520.587 | 61.997 |
| *Fchsd2* | -7.496 | 86.324 | 1.179 | 647.033 | 2.550 |
| *Naaa* | -7.574 | 200.658 | 1.640 | 1520.239 | 34.984 |
| *Cxx1c* | -7.700 | 62.578 | 0.293 | 492.805 | 103.302 |
| *Myo9a* | -7.899 | 82.853 | 8.502 | 653.389 | 55.378 |
| *Wdfy4* | -8.322 | 54.694 | 1.326 | 456.649 | 38.620 |
| *Zc3h12c* | -8.339 | 75.474 | 1.321 | 629.493 | 17.648 |
| *H2-DMb2* | -8.561 | 67.368 | 3.166 | 579.615 | 63.513 |
| *Cd86* | -8.666 | 84.368 | 1.793 | 731.713 | 33.724 |
| *Myo9a* | -9.705 | 109.991 | 9.023 | 1063.907 | 3.061 |
| *P2ry14* | -10.457 | 69.319 | 6.587 | 722.152 | 27.864 |
| *Cadm1* | -10.620 | 61.179 | 1.103 | 649.668 | 6.609 |
| *Fmnl2* | -11.584 | 116.254 | 11.221 | 1340.514 | 17.331 |
| *Btla* | -12.166 | 74.001 | 1.301 | 900.149 | 0.431 |
| *Flt3* | -13.114 | 107.580 | 12.807 | 1400.773 | 12.478 |
| *H2-Ob* | -13.854 | 65.471 | 3.630 | 905.658 | 5.029 |
| *Cd24a* | -13.964 | 91.100 | 7.591 | 1267.695 | 9.623 |
| *Ifi205* | -20.415 | 112.100 | 11.276 | 2276.985 | 8.050 |
| *Adam23* | -22.447 | 62.837 | 5.703 | 1404.960 | 26.830 |
| *A530099J19Rik* | -29.922 | 68.309 | 1.155 | 2044.525 | 61.354 |
| *Apol7c* | -30.727 | 129.958 | 51.582 | 3666.202 | 83.096 |
| *Dnase1l3* | -32.876 | 56.513 | 3.214 | 1855.880 | 59.356 |
| *Xcr1* | -36.979 | 50.499 | 1.581 | 1868.014 | 75.846 |

[+] Genes were selected which showed ≥ 5 fold change in signal value in either L-DC or $CD8^+$ cDC assessed in pairwise comparison.

* Data represent mean of duplicate samples (n=2).

**Table A.4 Genes specifically expressed between L-DC and $CD8^+$ cDC.**

| Gene Symbol | Fold-Change (L-DC vs. $CD8^-$ cD) $^+$ | L-DC | | $CD8^+$ cDC | |
|---|---|---|---|---|---|
| | | Mean* | Standard Error | Mean* | Standard Error |
| *Zeb2* | 14.837 | 548.052 | 67.119 | 36.668 | 0.693 |
| *Ifitm6* | 14.407 | 646.978 | 112.504 | 44.228 | 0.583 |
| *Gm9733* | 14.262 | 508.325 | 16.090 | 35.626 | 0.282 |
| *Gzmb* | 13.854 | 451.871 | 24.728 | 33.097 | 5.901 |
| *Ms4a4a* | 13.817 | 511.336 | 80.421 | 36.838 | 4.618 |
| *Atp1a3* | 13.665 | 528.196 | 5.192 | 38.733 | 2.487 |
| *L1cam* | 13.556 | 448.746 | 22.570 | 33.109 | 1.794 |
| *Gda* | 12.573 | 385.173 | 7.133 | 30.667 | 1.525 |
| *Sqrdl* | 12.326 | 328.967 | 23.265 | 27.184 | 5.500 |
| *Pltp* | 12.081 | 485.865 | 58.788 | 39.922 | 0.244 |
| *Lrp1* | 11.975 | 375.113 | 16.648 | 31.303 | 0.711 |
| *Lilra5* | 11.826 | 442.285 | 10.181 | 37.485 | 2.662 |
| *Ncr1* | 11.540 | 393.951 | 22.797 | 34.091 | 0.864 |
| *C3* | 11.306 | 425.896 | 13.786 | 37.653 | 0.302 |
| *Pla2g7* | 11.213 | 510.647 | 32.392 | 45.452 | 0.237 |
| *Tlr8* | 11.029 | 361.373 | 32.602 | 32.676 | 1.666 |
| *Abcd2* | 10.848 | 260.523 | 7.352 | 24.036 | 1.217 |
| *Nupr1* | 10.478 | 481.366 | 36.486 | 45.904 | 2.918 |
| *Hgf* | 10.139 | 278.901 | 5.863 | 27.518 | 0.972 |
| *Ikzf3* | 9.856 | 290.713 | 35.031 | 29.362 | 2.181 |
| *Fam46a* | 9.784 | 378.418 | 16.911 | 38.660 | 1.309 |
| *Krt80* | 9.635 | 423.455 | 0.465 | 43.950 | 0.235 |
| *Sepp1* | 9.454 | 326.572 | 47.610 | 34.184 | 0.839 |
| *Gm5150* | 9.449 | 367.228 | 82.481 | 37.895 | 1.292 |
| *A630033H20Rik* | 9.332 | 320.050 | 30.498 | 34.168 | 1.417 |
| *Irak3* | 9.060 | 264.136 | 8.938 | 29.259 | 2.663 |
| *Pparg* | 8.974 | 421.427 | 17.184 | 46.938 | 1.276 |
| *Abca9* | 8.492 | 234.244 | 11.094 | 27.566 | 0.790 |
| *Klra8* | 8.341 | 236.170 | 3.963 | 28.324 | 0.895 |
| *Fcgr3* | 8.200 | 300.711 | 28.094 | 36.512 | 0.215 |
| *Zfyve9* | 8.196 | 308.588 | 0.476 | 37.663 | 0.892 |
| *Fam55d* | 8.157 | 251.301 | 1.203 | 30.864 | 1.892 |
| *Arhgef3* | 7.919 | 387.354 | 66.081 | 48.211 | 1.141 |
| *Mgst1* | 7.917 | 166.937 | 11.405 | 21.039 | 0.326 |
| *S1pr5* | 7.904 | 303.395 | 4.288 | 38.477 | 2.729 |
| *Plxnb2* | 7.784 | 287.536 | 21.735 | 36.948 | 2.883 |
| *Mdm1* | 7.713 | 260.629 | 0.366 | 33.795 | 0.634 |
| *Ccl9* | 7.643 | 328.078 | 28.768 | 42.763 | 0.557 |
| *Gimap4* | 7.342 | 289.814 | 26.903 | 39.306 | 0.373 |
| *Svil* | 7.290 | 256.716 | 10.133 | 35.245 | 2.054 |
| *Trem3* | 7.262 | 357.258 | 13.473 | 49.163 | 0.101 |
| *Cd300lb* | 7.242 | 218.947 | 2.567 | 30.237 | 0.582 |
| *Ccdc125* | 6.898 | 197.052 | 2.197 | 28.629 | 1.895 |
| *Trem1* | 6.609 | 211.974 | 11.743 | 32.028 | 0.462 |
| *Gstm1* | 6.574 | 212.010 | 26.095 | 32.115 | 2.644 |
| *Rhoq* | 6.467 | 256.344 | 4.777 | 39.630 | 0.193 |
| *Tmem26* | 6.400 | 244.988 | 24.831 | 38.142 | 2.108 |
| *Xylt1* | 6.398 | 273.414 | 24.012 | 42.585 | 1.186 |
| *Rtp4* | 6.380 | 197.046 | 9.031 | 30.881 | 1.326 |
| *Pros1* | 6.351 | 179.988 | 24.135 | 28.108 | 1.130 |
| *Apoc2* | 6.340 | 240.037 | 11.321 | 37.820 | 0.295 |
| *Hp* | 6.313 | 182.405 | 43.577 | 28.123 | 1.913 |
| *Tmem71* | 6.311 | 151.394 | 20.686 | 23.769 | 0.444 |
| *Fpr1* | 6.308 | 169.278 | 2.425 | 26.835 | 0.312 |

| | | | | | |
|---|---|---|---|---|---|
| *Thbd* | 6.226 | 211.722 | 25.817 | 33.902 | 3.149 |
| *S100a6* | 6.167 | 219.687 | 25.302 | 35.415 | 1.409 |
| *Cd84* | 6.158 | 228.672 | 10.700 | 37.366 | 4.491 |
| *Rbpms* | 6.026 | 288.010 | 12.871 | 48.001 | 4.910 |
| *Tbc1d2b* | 5.971 | 191.380 | 0.900 | 32.084 | 1.493 |
| *Csf3r* | 5.969 | 163.212 | 1.153 | 27.365 | 1.077 |
| *Map3k15* | 5.930 | 247.350 | 7.442 | 41.866 | 3.836 |
| *B4galt5* | 5.804 | 270.397 | 21.621 | 46.762 | 5.476 |
| *Cyp4f18* | 5.798 | 195.922 | 7.464 | 33.830 | 2.110 |
| *Abi3* | 5.691 | 188.720 | 13.844 | 33.098 | 1.328 |
| *Gcnt2* | 5.659 | 201.374 | 9.994 | 35.548 | 0.638 |
| *Ms4a6d* | 5.547 | 158.618 | 7.103 | 28.609 | 1.567 |
| *Rap1gap2* | 5.470 | 212.381 | 9.855 | 38.794 | 0.906 |
| *6430548M08Rik* | 5.397 | 215.089 | 13.036 | 39.859 | 2.514 |
| *Pdlim1* | 5.343 | 244.698 | 2.334 | 45.881 | 2.751 |
| *Alox5* | 5.338 | 176.833 | 3.283 | 33.130 | 0.641 |
| *Ttc39a* | -5.281 | 41.159 | 1.918 | 217.696 | 15.663 |
| *Amica1* | -5.347 | 38.433 | 1.812 | 206.023 | 17.717 |
| *Trpm2* | -5.403 | 44.730 | 0.361 | 244.203 | 35.127 |
| *Frmd4b* | -5.486 | 43.374 | 2.417 | 237.666 | 6.732 |
| *Gca* | -5.498 | 45.694 | 2.830 | 252.937 | 33.173 |
| *Fam149a* | -5.810 | 27.369 | 1.392 | 159.397 | 13.597 |
| *Fnip2* | -6.095 | 42.679 | 5.517 | 259.547 | 28.938 |
| *Actn1* | -6.103 | 34.869 | 2.537 | 212.299 | 5.480 |
| *Met* | -6.298 | 35.512 | 2.706 | 223.965 | 20.668 |
| *Cxcl16* | -6.382 | 42.028 | 0.692 | 268.406 | 11.225 |
| *Ppef2* | -6.640 | 32.921 | 0.067 | 218.863 | 10.681 |
| *Fndc7* | -6.643 | 29.337 | 0.427 | 194.899 | 4.072 |
| *Nek6* | -6.724 | 34.587 | 2.361 | 232.055 | 4.099 |
| *H2-Oa* | -6.780 | 32.437 | 0.130 | 221.097 | 22.887 |
| *Cdon* | -6.952 | 41.034 | 2.449 | 284.776 | 0.732 |
| *Rab30* | -7.135 | 42.721 | 0.268 | 304.795 | 0.546 |
| *Clec1a* | -7.266 | 29.651 | 0.328 | 215.436 | 2.336 |
| *Itgae* | -7.569 | 29.068 | 0.032 | 221.400 | 24.737 |
| *Rnf144b* | -7.821 | 33.829 | 4.037 | 264.515 | 31.007 |
| *Ece1* | -7.880 | 47.285 | 0.344 | 374.364 | 36.515 |
| *Plekha5* | -8.317 | 42.413 | 6.056 | 349.171 | 6.188 |
| *Fam40b* | -8.470 | 30.717 | 1.708 | 262.130 | 35.061 |
| *Ciita* | -8.991 | 43.440 | 2.971 | 391.061 | 33.038 |
| *Gatm* | -9.527 | 32.792 | 2.301 | 311.982 | 14.869 |
| *Kmo* | -10.016 | 47.450 | 3.216 | 474.173 | 1.057 |
| *Adam19* | -10.264 | 40.698 | 1.667 | 418.164 | 25.395 |
| *Gcet2* | -10.314 | 30.685 | 0.161 | 316.697 | 11.734 |
| *Pdia5* | -10.391 | 34.510 | 2.207 | 360.117 | 40.376 |
| *Adam8* | -10.538 | 45.131 | 0.709 | 476.002 | 21.794 |
| *Hepacam2* | -11.851 | 23.649 | 1.339 | 280.052 | 11.621 |
| *Kit* | -12.288 | 45.381 | 3.567 | 556.738 | 30.154 |
| *Cacna1e* | -13.444 | 34.281 | 0.870 | 461.562 | 27.567 |
| *Ly75* | -13.465 | 44.834 | 0.452 | 603.775 | 10.825 |
| *Tlr3* | -14.975 | 30.232 | 1.223 | 452.356 | 2.728 |

[+] Genes were selected which showed between 5 to 15 fold change in signal value where one subset has signal value $\leq 50$ and the comparison subset has a signal value $\geq 150$, in either L-DC or $CD8^+$ cDC assessed in pairwise comparison.

* Data represent mean of duplicate samples (n=2).

## Table A.5 Genes specifically expressed L-DC and CD8$^-$ cDC.

| Gene Symbol | Fold-Change (L-DC vs. CD8$^-$ cDC)$^+$ | L-DC | | CD8$^-$ cDC | |
|---|---|---|---|---|---|
| | | Mean* | Standard Error | Mean* | Standard Error |
| *Gm10673* | 7.387 | 272.591 | 56.193 | 36.702 | 7.355 |
| *Fcgr3* | 7.119 | 300.711 | 40.598 | 42.165 | 3.019 |
| *Irak3* | 6.566 | 264.136 | 22.644 | 40.625 | 5.823 |
| *Map3k15* | 6.537 | 247.350 | 9.595 | 37.823 | 0.491 |
| *Hp* | 6.532 | 182.405 | 36.805 | 27.146 | 1.271 |
| *Tmem26* | 6.344 | 244.988 | 38.620 | 38.677 | 4.480 |
| *Mdm1* | 6.070 | 260.629 | 15.696 | 43.076 | 3.419 |
| *Apoc2* | 6.045 | 240.037 | 10.652 | 39.798 | 3.288 |
| *Fpr1* | 5.936 | 169.278 | 11.785 | 28.583 | 2.002 |
| *Thbd* | 5.904 | 211.722 | 22.901 | 35.616 | 1.195 |
| *Xylt1* | 5.803 | 273.414 | 32.386 | 46.949 | 1.091 |
| *Cd300lb* | 5.783 | 218.947 | 26.248 | 38.000 | 3.319 |
| *Svil* | 5.765 | 256.716 | 16.560 | 44.520 | 1.425 |
| *Trem1* | 5.757 | 211.974 | 20.345 | 36.820 | 2.085 |
| *Pros1* | 5.710 | 179.988 | 20.266 | 31.242 | 0.695 |
| *Ccdc125* | 5.227 | 197.052 | 15.631 | 37.781 | 2.532 |
| *Gstm1* | 5.092 | 212.010 | 16.306 | 41.796 | 6.312 |
| *Lrrc16a* | -5.055 | 35.121 | 73.789 | 177.984 | 16.044 |
| *Ptpn3* | -5.076 | 30.921 | 2.082 | 157.520 | 13.583 |
| *Ptpn3* | -5.165 | 47.759 | 7.073 | 246.474 | 10.867 |
| *Dennd3* | -5.320 | 37.311 | 4.596 | 199.139 | 16.767 |
| *Ctnnd2* | -5.372 | 33.902 | 1.754 | 182.360 | 12.929 |
| *Flnb* | -5.546 | 48.463 | 6.019 | 270.441 | 29.982 |
| *Bub1* | -5.601 | 34.466 | 5.957 | 195.833 | 36.781 |
| *Card11* | -5.850 | 36.686 | 2.685 | 214.530 | 18.356 |
| *Tns1* | -6.049 | 32.084 | 2.670 | 194.485 | 12.789 |
| *Mical3* | -6.091 | 46.421 | 5.960 | 282.759 | 12.329 |
| *Clec9a* | -6.333 | 45.057 | 3.686 | 285.108 | 36.356 |
| *Gpm6b* | -6.405 | 34.712 | 5.485 | 223.304 | 21.383 |
| *Tbc1d4* | -6.438 | 42.156 | 3.074 | 272.840 | 28.020 |
| *Siglecg* | -6.908 | 49.420 | 4.720 | 338.777 | 11.596 |
| *St8sia1* | -7.381 | 29.334 | 8.984 | 217.773 | 23.448 |
| *B4galt6* | -7.506 | 44.314 | 6.252 | 333.803 | 32.710 |
| *Cdon* | -7.539 | 41.034 | 2.337 | 309.066 | 12.666 |
| *Ciita* | -7.679 | 43.440 | 2.430 | 332.968 | 10.191 |
| *Ptpn3* | -7.950 | 33.400 | 4.627 | 266.634 | 30.051 |
| *1300002K09Rik* | -7.991 | 41.162 | 3.429 | 329.781 | 24.192 |

$^+$ Genes were selected which showed between 5 to 8 fold change in signal value where one subset has signal value $\leq 50$ and the comparison subset has a signal value $\geq 150$, in either L-DC or CD8$^-$ cDC assessed in pairwise comparison.

* Data represent mean of duplicate samples (n=2).

# Publications

**Publication relating to this thesis:**

O'Neill, H.C., Griffiths, K.L., Periasamy, P., Hinton, R.A., Petvises, S. Hey, Y.Y. and Tan, J.K. 2014. Spleen stroma maintains progenitors and supports long-term hematopoiesis. *Current Stem Cell Research & Therapy*, 9, 354-363.

Hey, Y.Y. and O'Neill, H.C. 2012. Murine spleen contains a diversity of myeloid and dendritic cells distinct in antigen presenting function. *Journal of Cell Molecular Medicine*, 16(11), 2611-9.

O'Neill, H.C., Griffiths, K.L., Periasamy, P., Hinton, R.A., Hey, Y.Y., Petvises, S. and Tan, J.K. 2011. Spleen as a site for haematopoiesis of a distinct antigen presenting cell type. *Stem Cells International*, 2011:954275.

Tan, J.K., Quah, B.J., Griffiths, K.L., Periasamy, P., Hey, Y.Y. and O'Neill, H.C. 2011. Identification of a novel antigen cross-presenting cell type in spleen. *Journal of Cell Molecular Medicine*, 15(5), 1189-99.

**In preparation:**

Papathanasiou, P., Petvises, S., Hey, Y.Y., Perkins, A. and O'Neill, H.C. Impact of the *c-Myb*$^{E308G}$ mutation on myelopoiesis in relation to dendritic cell development. *In preparation.*

Hey, Y.Y. and O'Neill, H.C. 2014. Redefining the splenic dendritic and myeloid subsets. *In preparation.*

Hey, Y.Y. and O'Neill, H.C. 2014. Characterisation of a novel dendritic-like cell type in spleen. *In preparation.*

Hey, Y.Y. and O'Neill, H.C. 2014. Molecular characterisation of splenic dendritic and myeloid subsets. *In preparation.*

Hey, Y.Y. and O'Neill, H.C. 2014. Development of a novel dendritic-like cell subset in spleen from endogenous splenic progenitors. *In preparation.*